

Engineering Mammalian Cell Line to Improve Sialylation

by

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Bachelor of Chemical Engineering
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SUBMITTED TO THE DEPARTMENT OF CHEMICAL ENGINEERING
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN CHEMICAL ENGINEERING
at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

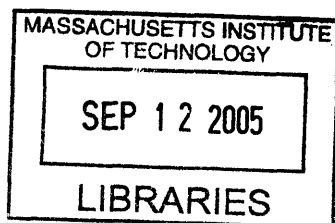
SEPTEMBER 2005

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ABSTRACT

One of the key problems faced by many biotechnology companies is the cleavage of terminal sialic acid on the glycans of the therapeutic glycoproteins. This is caused by the degradative action of sialidase released to supernatant when the cell starts to die. This phenomenon is undesirable because the loss of terminal sialic acid results in a product which is rapidly removed from the plasma by the interaction with asialoglycoprotein receptors in the liver. Many studies have been done in this area for decades and no general approach has been produced thus far.

In this study, RNA interference is utilized as a genetic approach to knock down the activity of sialidase which is responsible for cleaving terminal sialic acid. At the first stage of the studies, 21-nt double stranded siRNA sequences capable of knocking down sialidase are identified. The best sialidase siRNA sequence transiently knocks down sialidase mRNA by 9 folds and accompanied by a 4 fold reduction in sialidase activity. The most potent sialidase siRNA was located in UTR region and did not follow the widely-used Tuschl's rule.

At the second stage of the studies, a siRNA sequence is integrated into CHO cells using a plasmid with a drug selection marker to produce stable cell lines. It is found that the Pol III promoter is not strong enough to generate sialidase siRNAs. The modified CMV promoter is more appropriate for knocking down sialidase activity as clones with over 50% sialidase activity reduction can be isolated. We have isolated stable clones with over 60% sialidase knock down during the course of the cell cultivation. Growth rate and glycoprotein specific productivity of stable clones with reduced sialidase activity are not affected by siRNA activity or reduced sialidase expression. Glycan site occupancy of IFN γ produced by stable clones remains relatively unchanged. Two of the stable clones successfully maintain constant sialic acid content of IFN γ during prolonged cell culture even though cells are dying during these periods while the parent cell line loses the sialic acid at the rate of 0.05 mole sialic acid /mole IFN γ /day. This result is comparable to when sialidase inhibitor is used to deactivate sialidase. Microheterogeneity analysis reconfirms the consistent fraction of asialo, monosialyl, and bisialyl form of IFN γ for cell lines with reduced sialidase level during prolonged cell culture. On the other hand, parent cells are found to have more asialo and monosialyl form of IFN γ as the cells dies demonstrating the effect of sialidase release on glycoproteins during prolonged cell culture. Maximal sialic acid content during growth phase is found to be slightly altered by sialidase knock down. This could be due to clonal variation of parent cells or due to sialic acid salvage pathway disruption by reduced sialidase activity.

At the third stage of the studies, we develop a GFP-based method to rapidly and effectively isolate cells which express high amounts of sialidase siRNA. Subpopulations of CHO cells with

a high level of mean fluorescence intensity have lower sialidase mRNA level and activity. This implies a positive correlation between GFP fluorescence intensity and siRNA generated to silence sialidase. For similar fluorescent intensity, cells transfected with GFP-based Pol II-driven plasmid exhibits sialidase knock down 1-3 folds stronger than those transfected with GFP-based Pol III-driven plasmid.

We have successfully knocked down sialidase using a siRNA approach and produced not only stable cell line, but also functional and viable cell where cell growth is not affected by sialidase knock down. This method is a generic method that can be adopted by any biotech companies to reduce the sialidase degradative activity, producing a more consistent protein quality over the cell cultivation.

Thesis Supervisor: Daniel I. C. Wang
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ACKNOWLEDGEMENTS

There are so many people that have helped me technically and non-technically during the progress of this thesis. First, I would like to acknowledge Dr. Daniel I.C. Wang for all his support towards my PhD thesis. I very much appreciate the freedom to do research without worrying about lack of funding. His direct and honest advice is a trademark of his that has pushed me beyond my limits on achieving my research milestones. I sincerely appreciate his effort to provide me with invaluable resources needed to finish up this thesis and his generous time to chat with me regarding career planning.

My thesis committee members have been extremely helpful towards my research progress. Prof. Phil Sharp has been influential in helping me begin with RNAi work. His generous time and kind introduction to his group members have certainly benefited me in learning the biological aspect of my research. Prof. Greg Stephanopoulos and Prof. Barbara Imperiali also deserve special recognition for their advice during thesis committee meetings and class interactions. Although Prof. Kris Prather is not officially one of my thesis committee members, she has been very kind with her advices on her industrial/academia perspective on various research issues.

I acknowledge significant financial support from Motorola Corporation that made my research possible. MIT Technology and Development Program and Ms. Pat Vargas have been very generous in administering this research grant to Dr Wang. To show my appreciation to Motorola, I decided to use Motorola Razr V3 cell phone.

Life in the lab can be frustrating and I am very blessed to have the staff of MIT and BPEC to deal with the administrative work. Susan Lanza, Michelle Berry, Dan Darling, Suzanne Easterly, Cathy Green, Nils Nordal, and Aran Parillo have made it possible for me to work without worrying about receipts and paper works. Susan, I really cherish your attention to Prof. Wang's group. The breakfast that you provided us and the fantastic home-made cake that you baked during our birthdays are something that every research group at MIT is jealous about. As Bernat always pointed out, social life in our group exists because of you. Niki and I agree your cheesecake should be sold online to compete with the Cheesecake Factory and Finale.

The nature of research is collaborative and I am very blessed with the gracious help of numerous people at MIT and Singapore's Bioprocessing Technology Institute (BTI). John Doench and Dr. Carl Novina from Prof. Sharp's group have been very patient with my basic questions on RNAi. Special thanks to Niki Wong for her help with my interferon-gamma samples. Our daily discussions on various research topics during your last semester here have broadened my horizon. I wish I had known you earlier.

It is especially important to express my sincere and grateful appreciations to the various individuals in Singapore. My deep appreciations goes to Professor Miranda Yap, Executive Director of the Bioprocessing Technology Institute (BTI), A*STAR, who graciously allowed her staff to assist me on the analytical characterizations of the glycan structures. Without the help from BTI, this thesis could not have been achieved in the time-frame for my dissertation.

The research teams in BTI led by Dr. Goh Lin Tang and Dr. May Lee Lee have been instrumental in assisting me with the superb analytical works that made this thesis possible. I am very grateful with all your patience to deal with my numerous samples.

Past and present members in Dr. Daniel Wang's group have contributed to this thesis in different ways. Drs. Yin Jin and Peng Jiao were very patient in teaching me basic molecular biology and mammalian cell culture. Kelly Kral shared frustration with me when the CHO cells were unhappy for no reason. Drs. Brian Baynes, Andre Ditsch, and Stephen Fox have shared their time listening to my research problems, career concerns, and panic attack during thesis write-ups. Bernat Olle has shared excitement when I got good data after months and months of late night work. My undergraduate students (UROPs), Peter Miller and Fik Brushett, enabled me to occasionally have a good night sleep by working extremely hard with me. Many graduate students complained they had useless UROPs but I am not one of them because of the excitement that we shared from pages of data. I have learned a lot from both of you and hope that you have learned as much from me.

Beyond Prof. Daniel Wang's group members, there are many of my colleagues and friends that deserve special acknowledgements. Keith Tyo should get gold medal for providing me with many biology-related suggestions, allowing me to use many machines in Prof. Stephanopoulos' lab, editing heaps of my badly written papers, reading my boring literature reviews, and providing Easter dinner with an organic ham! Michael Rappel kindly shared his valuable time to edit a few chapters of my thesis without complaints. Emily Larson and Megan Whittemore were very kind to deal with safety issues in BPEC lab. Thien Nguyen, Kirill Titievsky, Meiliana Tjandra, and Mario Choi have provided significant support when things go wrong. Their companies during my sugar cravings period (i.e. cheesecake, tiramisu, and warm chocolate cake) are deeply appreciated.

Finally, I am very thankful for the kind love and prayers from my parents, brother, and sister kilometers away from Boston. Although they are not familiar with what I am working on for my PhD thesis, their support has enabled me to never give up when tough challenges arise during these past four years.

TABLE OF CONTENTS

ABSTRACT.....	3
ACKNOWLEDGEMENTS	5
TABLE OF CONTENTS	7
LIST OF FIGURES.....	9
LIST OF TABLES.....	12
1. INTRODUCTION.....	15
1.1 BACKGROUND.....	15
1.2 MOTIVATION.....	16
1.3 THESIS OBJECTIVES	17
1.4 THESIS ORGANIZATION.....	17
2. LITERATURE REVIEW	19
2.1 PROTEIN GLYCOSYLATION OVERVIEW	19
2.1.1 <i>Roles of glycosylation in therapeutic proteins.....</i>	<i>19</i>
2.1.2 <i>Glycosylation and glycoforms.....</i>	<i>23</i>
2.1.3 <i>Choice of host cells for glycoproteins.....</i>	<i>31</i>
2.1.4 <i>Factors affecting glycosylation in mammalian cell culture.....</i>	<i>40</i>
2.2 TERMINAL SIALIC ACID: ROLE, SYNTHESIS, AND DEGRADATION.....	46
2.2.1 <i>Role of terminal sialic acid as biological mask.....</i>	<i>46</i>
2.2.2 <i>Sialidase and glycoprotein.....</i>	<i>50</i>
2.2.3 <i>Effect of cell culture conditions on sialylation of glycoproteins.....</i>	<i>52</i>
2.2.4 <i>Genetic engineering methods to improve sialylation.....</i>	<i>58</i>
2.3 RNA INTERFERENCE OVERVIEW	60
2.3.1 <i>Mechanism of RNA interference (RNAi).....</i>	<i>60</i>
2.3.2 <i>Transient and stable silencing by RNAi.....</i>	<i>63</i>
2.3.3 <i>RNAi and glycoengineering of CHO cells</i>	<i>64</i>
2.4 MODEL SYSTEM: RECOMBINANT HUMAN INTERFERON GAMMA (IFN γ) PRODUCED BY CHINESE HAMSTER OVARY CELLS.	65
3. MATERIALS AND METHODS.....	67
3.1 CELL CULTURE	67
3.1.1 <i>Cell lines</i>	<i>67</i>
3.1.2 <i>Culture medium, transformation, and maintenance</i>	<i>68</i>
3.1.3 <i>CHO Cell Bank Maintenance</i>	<i>70</i>
3.2 SIALIDASE siRNA METHOD.....	71
3.2.1 <i>Optimization of transfection condition</i>	<i>71</i>
3.2.2 <i>Sialidase siRNA design</i>	<i>72</i>
3.2.3 <i>Preparation of siRNA duplexes.....</i>	<i>73</i>
3.2.4 <i>Sialidase siRNA transfection and RNA collection</i>	<i>73</i>
3.2.5 <i>Sialidase activity assay</i>	<i>74</i>
3.2.6 <i>Thermodynamic analysis of siRNA sequences.....</i>	<i>75</i>

3.3	RNA ANALYSIS	75
3.3.1	<i>Total RNA isolation</i>	75
3.3.2	<i>RNA quantification</i>	76
3.3.3	<i>First-strand cDNA synthesis</i>	77
3.3.4	<i>RT-PCR assay</i>	77
3.3.5	<i>Real time PCR quantification (qPCR) assay</i>	78
3.4	STABLE CELL LINE CREATION	83
3.4.1	<i>Plasmid design and transfection</i>	83
3.4.2	<i>Screening for Low Sialidase Activity CHO Clones</i>	88
3.5	IFN- γ ANALYTICAL METHODS	89
3.5.1	<i>Purification and quantification of IFN-γ</i>	89
3.5.2	<i>Sialic acid content analysis of IFN-γ</i>	89
3.5.3	<i>Glycan site occupancy analysis of IFN-γ</i>	90
3.5.4	<i>Microheterogeneity analysis of IFNγ</i>	91
3.6	OTHER ANALYTICAL METHODS.....	94
3.6.1	<i>Determination of IFN-γ concentration</i>	94
3.6.2	<i>Glucose, glutamine, ammonia, and lactate Concentration Measurement</i>	94
3.7	CONSTRUCTION OF GFP-BASED shRNA PLASMID	95
3.7.1	<i>GFP-based screen for cells expressing high copy numbers of pol III-driven sialidase siRNA plasmid construct</i>	95
3.7.2	<i>GFP-based screen for cells expressing high copy numbers of pol II-driven sialidase siRNA plasmid construct</i>	96
4.	INITIAL INVESTIGATION ON EXPLOITING RNA INTERFERENCE	
	TECHNIQUE TO REDUCE SIALIDASE DEGRADATIVE ACTIVITY	101
4.1	INTRODUCTION	101
4.2	RESULTS AND DISCUSSIONS.....	104
4.3	CONCLUSIONS.....	136
5.	RNA INTERFERENCE OF SIALIDASE IMPROVES GLYCOPROTEIN SIALIC	
	ACID CONTENT CONSISTENCY.....	139
5.1	ABSTRACT	139
5.2	INTRODUCTION	140
5.3	RESULTS	150
5.4	DISCUSSIONS	198
5.5	CONCLUSIONS.....	201
5.6	ACKNOWLEDGMENTS	201
6.	CONCLUSIONS AND RECOMMENDATIONS.....	203
6.1	THESIS CONCLUSIONS.....	203
6.2	RECOMMENDATIONS FOR FUTURE WORK	205
7.	REFERENCES.....	209

LIST OF FIGURES

FIGURE 2.1 STRUCTURES OF N-LINKED GLYCOPROTEIN.....	24
FIGURE 2.2 CORE STRUCTURES OF O-LINKED GLYCOSYLATION.....	25
FIGURE 2.3 MACROHETEROGENEITY OF INTERFERON- γ GLYCOSYLATION	27
FIGURE 2.4 ASSEMBLY OF N-LINKED GLYCANS IN MAMMALIAN CELLS	29
FIGURE 2.5 SCHEMATIC DIAGRAM OF SIALIC ACID METABOLISM	53
FIGURE 2.6 RNA INTERFERENCE (RNAi) MECHANISM FOR GENE-SPECIFIC mRNA DEGRADATION	62
FIGURE 3.1 REAL TIME PCR FLUORESCENCE VERSUS PCR CYCLE NUMBER.	80
FIGURE 3.2 THRESHOLD CYCLE (Ct) AS A FUNCTION OF DNA STANDARDS CONCENTRATIONS FOR SIALIDASE AND β -ACTIN.....	81
FIGURE 3.3 MELTING CURVE ANALYSIS OF SIALIDASE AND β -ACTIN STANDARDS.....	82
FIGURE 3.4 VECTOR MAP OF pSILENCER WITH U6 PROMOTER	84
FIGURE 3.5 VECTOR MAP OF pSILENCER WITH H1 PROMOTER	85
FIGURE 3.6 VECTOR MAP OF pSILENCER WITH MODIFIED CMV PROMOTER.....	86
FIGURE 3.7 HIGH PERFORMANCE ANION EXCHANGE CHROMATOGRAPHY SPECTRA OF INTERFERON- GAMMA GLYCANS. IN EVERY RUN, RAFFINOSE WAS USED AS INTERNAL STANDARD.....	93
FIGURE 3.8 SCHEMATIC DIAGRAM OF SITE SPECIFIC RECOMBINATION PROCESS TO ENGINEER POL III-DRIVEN shRNA-GFP PLASMID BEARING DRUG RESISTANCE MARKER.....	97
FIGURE 3.9 SCHEMATIC DIAGRAM OF POL II-DRIVEN pSILENCER 4.1-CMV PLASMID THAT CONTAINS SIALIDASE HAIRPIN RNA TEMPLATE AND GFP SEQUENCE.	99
FIGURE 4.1 EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE OF SIALIDASE DESIALYLATION REACTION.....	105
FIGURE 4.2 LINWEAVER-BURK PLOT OF IN VITRO SIALIDASE DESIALYLATION REACTION AT VARIOUS IFN γ GLYCOPROTEIN CONCENTRATIONS	107
FIGURE 4.3 EVALUATION OF VARIOUS TRANSFECTION REAGENTS EFFECTIVENESS IN DELIVERING FLUORESCHEIN-LABELED NON-SILENCING DOUBLE STRANDED RNA	111
FIGURE 4.4 THE EFFECT OF INCREASING THE DOSAGE OF LIPOFECTAMINE 2000 TRANSFECTION REAGENT ON THE DELIVERY OF FLUORESCHEIN-LABELED siRNA	114
FIGURE 4.5 mRNA QUANTIFICATION OF CELLS TRANSFECTED WITH SIALIDASE siRNA TABULATED IN TABLE 4.2.	117
FIGURE 4.6 SIALIDASE ACTIVITY OF CHO-IFN γ CELLS TRANSFECTED WITH SIALIDASE siRNA TABULATED IN TABLE 4.2.	118
FIGURE 4.7 REDUCTION IN SIALIDASE mRNA LEVEL BY DOUBLE STRANDED RNA INTERFERENCE MECHANISM CORRESPONDS TO THE DECREASE IN SIALIDASE ACTIVITY.....	119
FIGURE 4.8 MODEL FOR RISC ASSEMBLY BASED ON SCHWARTZ' ASYMMETRY RULE. THIS FIGURE IS ADAPTED FROM SCHWARTZ ET AL. (2003).....	124
FIGURE 4.9 THERMODYNAMIC ANALYSIS UTILIZING SCHWARTZ ET AL.'S ASYMMETRICAL RULE DID NOT ADEQUATELY PREDICT REDUCTION IN SIALIDASE ACTIVITY CONTRIBUTED FROM SMALL INTERFERING RNA.....	126

FIGURE 4.10 THERMODYNAMIC ANALYSIS UTILIZING KHVOROVA'S AVERAGE OF INTERNAL STABILITY ANALYSIS FOR POSITIONS 9-14 OF THE ANTISENSE STRAND (AIS) PREDICTION YIELDED THE OPPOSITE TREND SHOWN BY EXPERIMENTAL RESULTS.....	128
FIGURE 4.11 RT-PCR ANALYSIS OF INTERFERING CHO-IFN γ SIALIDASE mRNA USING siRNA SEQUENCE S1	130
FIGURE 4.12 RT-PCR ANALYSIS OF INTERFERING CHO-IFN γ SIALIDASE mRNA USING siRNA SEQUENCE S5	131
FIGURE 4.13 RT-PCR ANALYSIS OF INTERFERING CHO-IFN γ SIALIDASE mRNA USING siRNA SEQUENCE S6	132
FIGURE 4.14 SIALIDASE ACTIVITY REDUCTION AS A RESULT OF TRANSIENT CHO-IFN γ SIALIDASE mRNA SILENCING USING siRNA SEQUENCE S1, S5, AND S6.	133
FIGURE 4.15 RETRANSFECTION OF CHO CELLS WITH SIALIDASE siRNA RECOVERED THE siRNA ACTIVITY TO REDUCE SIALIDASE ACTIVITY IN VARIOUS CHO CELLS.....	135
FIGURE 4.16 INTERFERING SIALIDASE mRNA TO KNOCK DOWN SIALIDASE mRNA AND TO REDUCE SIALIDASE ACTIVITY IS UNIVERSAL TO VARIOUS CHO CELLS.	138
FIGURE 5.1 TRANSLATION OF SIALIDASE siRNA SEQUENCE FROM TRANSIENT TO STABLE SYSTEM.	144
FIGURE 5.2 MACROHETEROGENEITY AND SIALIC ACID QUANTIFICATION ANALYSIS OF PURIFIED INTERFERON GAMMA OBTAINED FROM PARENT CELLS AND SINGLE CELL CLONES TRANSFECTED WITH STABLE SIALIDASE siRNA SYSTEM.	145
FIGURE 5.3 CHARACTERIZATION OF INTERFERON GAMMA MICROHETEROGENEITY.	146
FIGURE 5.4 STABILITY ANALYSIS OF SIALIDASE SUPPRESSION ON <u>ATTACHED</u> SINGLE CELL CLONES TRANSFECTED WITH shRNA PLASMID UTILIZING U6 AND H1 PROMOTER	154
FIGURE 5.5 SIALIDASE ACTIVITY OF VARIOUS <u>ATTACHED</u> SINGLE CELL COLONIES RESULTED FROM TRANSFECTION OF SIALIDASE shRNA PLASMID UTILIZING MODIFIED CMV PROMOTER.	156
FIGURE 5.6 SIALIDASE ACTIVITY PROFILE FOR THREE <u>ATTACHED</u> SINGLE CELL CLONES TRANSFECTED WITH shRNA PLASMID CONTAINING MODIFIED CMV PROMOTER	158
FIGURE 5.7 STABILITY ANALYSIS OF SIALIDASE SUPPRESSION ON <u>ATTACHED</u> SINGLE CELL CLONES TRANSFECTED WITH shRNA PLASMID UTILIZING MODIFIED CMV PROMOTER: FLUORESCENCE ASSAY	160
FIGURE 5.8 STABILITY ANALYSIS OF SIALIDASE SUPPRESSION ON SINGLE CELL CLONES TRANSFECTED WITH shRNA PLASMID UTILIZING MODIFIED CMV PROMOTER: PCR ASSAY	161
FIGURE 5.9 VIABLE CELL DENSITIES OF <u>ATTACHED</u> SINGLE CELL CLONES TRANSFECTED WITH shRNA PLASMID UTILIZING MODIFIED CMV PROMOTER.....	163
FIGURE 5.10 GLYCOPROTEIN (IFN γ) PRODUCTIVITY OF <u>ATTACHED</u> SINGLE CELL CLONES TRANSFECTED WITH shRNA PLASMID UTILIZING MODIFIED CMV PROMOTER DURING BATCH CULTURE OF CHO-IFN- γ	166
FIGURE 5.11 SIALIC ACID CONTENT OF <u>ATTACHED</u> SINGLE CELL CLONES TRANSFECTED WITH shRNA PLASMID UTILIZING MODIFIED CMV PROMOTER.....	168
FIGURE 5.12 mRNA QUANTIFICATION OF STABLE <u>ATTACHED</u> SINGLE CELL CLONES TRANSFECTED WITH shRNA PLASMID UTILIZING MODIFIED CMV PROMOTER	170
FIGURE 5.13. GLYCAN SITE OCCUPANCY OF IFN γ FROM STABLY TRANSFECTED CLONE AND PARENT CELL	172

FIGURE 5.14 HIGH PERFORMANCE ANION EXCHANGE CHROMATROGRAPHY SPECTRA OF INTERFERON-GAMMA GLYCANS OBTAINED FROM CLONE S1E CELL LINES DURING GROWTH PHASE AND DEATH PHASE.	175
FIGURE 5.15 HIGH PERFORMANCE ANION EXCHANGE CHROMATROGRAPHY SPECTRA OF INTERFERON-GAMMA GLYCANS OBTAINED FROM PARENT CELL LINES DURING GROWTH PHASE AND DEATH PHASE.	176
FIGURE 5.16 MICROHETEROGENEITY ANALYSIS: EFFECT OF INHIBITING SIALIDASE VIA RNA INTERFERENCE TECHNIQUE ON SIALYLATION OF COMPLEX BIA TENNARY INTERFERON GAMMA GLYCANS IN BATCH CHO CELL CULTURE.....	178
FIGURE 5.17 SCHEMATIC DIAGRAM OF POSSIBLE ROLE OF SIALIDASE IN SALVAGE/RECYCLE PATHWAY OF SIALIC ACID.	183
FIGURE 5.18 FACS ANALYSIS OF CHO CELLS TRANSFECTED WITH POL III-DRIVEN SHRNA-GFP PLASMID BEARING DRUG RESISTANCE MARKER.....	186
FIGURE 5.19 MEAN GFP FLUORESCENCE INTENSITIES OF SELECTED SUBPOPULATIONS TRANSFECTED WITH POL III-DRIVEN GFP-BASED SHRNA PLASMID AND UNTRANSFECTED PARENT CELL LINES ARE COMPARED OVER THE COURSE OF CELL CULTURE.....	187
FIGURE 5.20 NORMALIZED SIALIDASE mRNA LEVEL OF SUBPOPULATIONS TRANSFECTED WITH POL III-DRIVEN GFP-BASED SHRNA PLASMID OBTAINED FROM FACS ANALYSIS ARE COMPARED WITH UNTRANSFECTED PARENT CELL LINES.	189
FIGURE 5.21 SIALIDASE ACTIVITY OF SUBPOPULATIONS TRANSFECTED WITH POL III-DRIVEN GFP-BASED SHRNA PLASMID OBTAINED FROM FACS ANALYSIS ARE COMPARED WITH UNTRANSFECTED PARENT CELL LINES	190
FIGURE 5.22 FLUORESCENCE MICROSCOPY IMAGES OF CHO CELLS TRANSFECTED WITH PEGFP-C1 PLASMID (A) AND WITH MODIFIED pSILENCER 4.1-CMV PLASMID (B) ARE COMPARED.....	192
FIGURE 5.23 FACS ANALYSIS OF CHO CELLS TRANSFECTED WITH POL II-DRIVEN SHRNA-GFP PLASMID BEARING DRUG RESISTANCE MARKER.....	193
FIGURE 5.24 MEAN GFP FLUORESCENCE INTENSITIES OF SELECTED SUBPOPULATIONS TRANSFECTED WITH POL II-DRIVEN GFP-BASED SHRNA PLASMID AND UNTRANSFECTED PARENT CELL LINES ARE COMPARED OVER THE COURSE OF CELL CULTURE.....	195
FIGURE 5.25 NORMALIZED SIALIDASE mRNA LEVEL OF SUBPOPULATIONS TRANSFECTED WITH POL II-DRIVEN GFP-BASED SHRNA PLASMID OBTAINED FROM FACS ANALYSES (FIGURE 5.23) ARE COMPARED WITH UNTRANSFECTED PARENT CELL LINES.	196
FIGURE 5.26 SIALIDASE ACTIVITY OF SUBPOPULATIONS TRANSFECTED WITH POL II-DRIVEN GFP-BASED SHRNA PLASMID OBTAINED FROM FACS ANALYSES (FIGURE 5.23) ARE COMPARED WITH UNTRANSFECTED PARENT CELL LINES.	197

LIST OF TABLES

TABLE 2.1 ENZYMES INVOLVED IN THE ASSEMBLY OF N-LINKED OLIGOSACCHARIDES.....	30
TABLE 2.2 STRUCTURE OF N-GLYCANS PRODUCED BY VARIOUS EXPRESSION SYSTEMS.....	39
TABLE 2.3 THE EFFECT OF SIALIC ACID REMOVAL ON THE CIRCULATORY HALF-LIFE TIME OF SELECTED GLYCOPROTEINS	49
TABLE 3.1 SEQUENCE OF 21-NUCLEOTIDE siRNA AND shRNA TEMPLATE DESIGNED TO TARGET SIALIDASE mRNA	87
TABLE 4.1 SIALIDASE KINETIC CONSTANTS ESTIMATED FROM REACTING CHO-IFN- γ CELL LYSATE WITH VARIOUS SIALYLATED SUBSTRATES	109
TABLE 4.2 SIALIDASE siRNA SEQUENCES DESIGNED USING TUSCHL'S RULE AND ARBITRARY CHOICES.	116
TABLE 4.3 THERMODYNAMIC ANALYSIS OF VARIOUS SIALIDASE siRNA SEQUENCES.....	125
TABLE 5.1 SIALIDASE ACTIVITY OF VARIOUS ATTACHED SINGLE CELL COLONIES RESULTED FROM TRANSECTION OF SIALIDASE shRNA PLASMID UTILIZING U6 PROMOTER.	151
TABLE 5.2 SIALIDASE ACTIVITY OF VARIOUS ATTACHED SINGLE CELL COLONIES RESULTED FROM TRANSECTION OF SIALIDASE shRNA PLASMID UTILIZING H1 PROMOTER.	152
TABLE 5.3 AVERAGE SPECIFIC GROWTH RATES FOR BATCH CULTURE OF ATTACHED SINGLE CELL CLONES TRANSFECTED WITH shRNA PLASMID COMPARED WITH PARENT CELLS.	164
TABLE 5.4 MICROHETEROGENEITY STRUCTURES OF INTERFERON GAMMA GLYCANS OBSERVED (+) UTILIZING HPAEC-PAD (AEC) AND MALDI-TOF ANALYSIS OF PERMETHYLATED GLYCANS (MS).	174
TABLE 5.5 BIA TENNARY GLYCAN STRUCTURES OF INTERFERON-GAMMA PRODUCED BY CLONE S1E AND PARENT CHO-IFN γ CELL LINES.	180

1. Introduction

1.1 Background

Recombinant therapeutic proteins from mammalian cells have proven to be effective in the treatment of various diseases. More importantly, biopharmaceuticals from mammalian cells possess advantages such as higher biological activity, increased solubility and extended circulatory *in vivo* half life. This is due to a post-translational modification process known as glycosylation that cannot be done properly by *E.coli* or yeast expression systems. In large scale production of mammalian cell glycoproteins there are various problems that need to be solved.

One problem generally faced by biopharmaceutical companies is the lack of protein quantity. Since protein production is strongly correlated to the viable cell number in the bioreactors, maintaining a high density of viable cells for prolonged time is one way to increase protein quantity. Many strategies have been proposed to reach higher cell density such as stoichiometric fed-batch culture or fed batch culture with substitution of glucose and glutamine for slowly metabolized nutrients (Xie, 1997; Altamirano et al., 2004). About 80% of cell death in standard serum free batch culture of CHO cells in suspension is caused by apoptosis (Goswami et al., 1999). Thus, genetic engineering approaches that either interfere with the activation of apoptosis-related genes or overexpress anti-apoptotic gene have been attempted by many researchers (reviewed in Arden and Betenbaugh, 2004).

Another common problem in biopharmaceuticals production is to maintain the homogeneity of protein quality. Although most glycoproteins with some degree of glycosylation heterogeneity

are accepted by FDA from mammalian cells, future work must be done to improve the consistency of glycosylation for each production phase. One contribution to the heterogeneity is the cleavage of terminal sialic acid on the glycans by the glycosidase enzymes. This phenomenon is undesired because the loss of terminal sialic acid results in a product which is rapidly removed from the plasma by the interaction with asialoglycoprotein receptors. Many studies in the past have focused on culture alterations which were not successful due to the non-specific nature of the strategy. With the advent of molecular biology, engineering mammalian cells to produce consistently sialylated glycoprotein is now possible.

1.2 Motivation

About 60% of therapeutic protein markets are glycoproteins with annual growth rate of 26% (Gerngross, 2004). With increasing demands of complex therapeutic proteins, there is no doubt that large scale mammalian cell culture must be performed to meet the demands. Producing high amount of glycoprotein is now possible with the better understanding of cell metabolism and genes pertinent to cell viability or cell death. Specific productivity of an optimized glycoprotein production process in 2004 is about 9 times higher than that in 1986 while product titer can reach as high as 5 g/L (Wurm, 2004). Nevertheless, the increase in therapeutic glycoprotein quantity must be balanced with a consistent (or better) glycoprotein quality. This is in accordance to the FDA requirement for consistent product quality (Liu, 1992). Cell death is still inevitable and this translates to the release of degradative enzymes as culture time increases. Thus, any attempts to improve protein quality by engineering cells with reduced degradative enzymes expression will enable the production of therapeutic proteins at prolonged culture times.

1.3 Thesis Objectives

The main goal of this thesis is to genetically engineer a mammalian cell line with improved glycoprotein quality consistency. The model system used was a Chinese Hamster Ovary (CHO) cell line that produced recombinant human interferon-gamma (IFN γ). Within this central goal, there are three objectives of this thesis. First, demonstrate that RNA interference (RNAi) is a viable method to transiently silence cytosolic sialidase. This will involve characterization of sialidase activity and verification of sialidase knock down at the mRNA level. Second, create a stable CHO cell line that continuously produces cytosolic sialidase small interfering RNA (siRNA). This will involve further characterization on the quantification of sialic acid content and on the microheterogeneity distribution of glycoproteins. A success in knocking down gene pertinent to sialic acid degradation should lead to consistent sialic acid content throughout various phase of cell culture. Third, establish a methodology to select engineered cells with reduced sialidase level if indeed sialidase knock down leads to increased sialic acid consistency. This will involve a GFP-based screening method in lieu of testing over 1000 clones for a desired cell line.

1.4 Thesis Organization

The thesis is divided into six chapters. Chapter 2 provides a literature review on glycosylation, sialylation, and basic RNA interference. In Chapter 3, the materials and methods for this study are explained in detail. Chapter 4 demonstrates the effectiveness of RNAi method to transiently silence sialidase activity in CHO-IFN- γ cells along with some other pertinent CHO cell lines. Thermodynamic analysis on cytosolic sialidase siRNA sequences is briefly explained. In Chapter 5 stable CHO cell lines with consistent reduced sialidase activity are created. Various

characterization methods are utilized to examine the improvement in glycoprotein quality along with a stability analysis of the transformed CHO cell lines. Chapter 5 also deals with the attempt to speed up the selection process of stable cell line by incorporating a GFP-based technique. Chapter 6 presents concluding remarks and recommendations for future research.

2. Literature Review

2.1 Protein Glycosylation Overview

2.1.1 Roles of glycosylation in therapeutic proteins

Glycosylation is a post-translational modification processes that can only be performed properly by eukaryotic cells (reviewed in Kornfeld and Kornfeld, 1985; Jenkins and Curling, 1994). It is the process of adding a variety of functional carbohydrate groups to the backbone of a peptide through certain amino acid consensus sequences. Proteins that contain these sugar groups are called glycoproteins and they exist in heterogeneous populations called glycoforms. The presence of carbohydrates on the backbone of polypeptides has been shown to affect many protein properties such as: solubility, stability, biological activity, immunogenicity, and pharmacokinetics (reviewed in Jenkins and Curling, 1994). In this section, the effect of glycosylation on the protein properties will be reviewed.

Glycosylation increases global protein stability (reviewed in Imperiali and O'Connor, 1999). Thermodynamic studies on ovomucoid demonstrated that glycosylated and non-glycosylated forms of protein had the same enthalpy (DeKoster and Robertson, 1997). Since the glycosylated form has increased free energy of unfolding, the stabilization of glycosylated protein was hypothesized to be due to entropic contribution. It was proposed that the existence of oligosaccharide helped to reduce the disorder of an unfolded protein.

Oligosaccharides attachment to polypeptide backbone provides thermal stabilization of the protein. In the circular dichroism (CD) spectroscopy and differential scanning microcalorimetry

(DSC) studies performed by Wang and co-workers, it was shown that carbohydrate removal of heavily glycosylated proteins led to melting point reduction of 1.3 – 2.8 °C (Wang et al., 1996). Nonglycosylated β -1,3/1,4-glucanases produced by *Escherichia coli* (*E. coli*) were much more heat labile as compared to the glycosylated counterparts produced by *Saccharomyces cerevisiae* (*S. cerevisiae*) (Olsen and Thomsen, 1991). Ovomucoid protein, when glycosylated, has a melting temperature that was 4.8°C higher than when unglycosylated (DeKoster and Robertson, 1997). There were two explanations on why glycosylation enhanced thermal stability of protein. First, carbohydrate moieties may form hydrogen bonds with the polypeptide backbones (Hecht et al., 1993; Woods et al., 1994). This explanation arose from the crystal structure study of glucose oxidase that showed that N-linked glycans formed hydrogen bonds with glutamic acid. Second, thermal stability might arise from steric interactions of carbohydrate with adjacent polypeptides (Rudd et al., 1994).

Glycosylation protects protein from proteases attack. Recombinant IFN γ proteins made by *E.coli* and baculovirus were shown to be very prone to crude granulocyte protease, purified elastase, cathepsin G, and plasmin degradation (Sareneva et al., 1995). On the other hand, fully glycosylated IFN γ had full or partial protection from the protease degradation. Cytokine granulocyte colony stimulating factor (G-CSF) produced by Chinese Hamster Ovary (CHO) cells was demonstrated to be more resistant to serum protease enzymes compared to the same protein produced by an *E.coli* expression system (Carter et al., 2004). Since CHO produced glycosylated protein, resistance towards protease degradation was concluded to be contributed by the existence of oligosaccharides on the proteins. NMR and X-ray crystallography studies on some other glycoproteins demonstrated that the overall 3D structure of protein was not affected by

glycosylation (Rudd et al., 1994). However, amide proton exchange rate experiments showed that there was increase rigidity in glycosylated proteins, which might lead to increased protease resistance.

Glycosylation affects the biological activity of a number of glycoproteins. Human interferon- β (hIFN β) produced by Chinese Hamster Ovary (CHO) cells were shown to be 10 times more active than the non-glycosylated counterpart produced by *E.coli* (Runkel et al., 1998). Angiotensin-converting enzyme (ACE_T) was devoid of enzymatic activity when it was produced by *E.coli* or by tunamycin-treated HeLa cells (Sadhukhan and Sen, 1996). Biopotency in rats of follicle stimulating hormone (FSH) was significantly affected by the number of glycans attached on FSH, although maximum elimination half life did not increase as significantly (Weenen et al., 2004). Mutation of Asn286 site where glycosylation occurred led to the loss of CLN2 enzymatic activity (Tsiakas et al., 2004). However, on some glycoproteins, the existence of oligosaccharides on the polypeptide backbone is not needed for full biological activity. For tissue plasminogen activator (tPA), the less glycosylated isoform possessed 20-30% greater activity as compared to the more glycosylated isoform (Berg et al., 1993). *In vitro* activity of IFN γ was demonstrated to be independent of the glycan existence since *E.coli*-produced IFN γ has full antiviral and anti-proliferative *in vitro* activity (Rinderknecht et al., 1984). Although N-glycosylation is important for proper folding of human sialyltransferase ST3Gall, none of the four N-glycans attachment sites in the catalytic domain was needed for enzymatic activity (Jeanneau et al., 2004). Therefore, biological activity of protein may or may not be affected by the presence of oligosaccharides.

Glycosylation affects the solubility of therapeutic glycoproteins. When N-linked glycans were removed from glycosylated erythropoietin (EPO), it was found that solubility of the protein declined significantly (Dordal et al., 1985). Oh-eda and coworkers (1990) discovered that the main difference between the glycosylated and deglycosylated form of human granulocyte colony stimulating-factor (hG-CSF) was that deglycosylated hG-CSF formed polymers at pH 7.4 and 8.0, which was the typical operating pH for bioreactor operations. Further, this polymerized deglycosylated hG-CSF formed insoluble aggregates which showed no biological activity. It was concluded that oligosaccharides in hG-CSF prevented polymerization of the factor allowing it to be biologically active. For hIFN β glycoprotein, a crystallographic study showed that carbohydrates shielded exposure of solvent with abnormally large number of hydrophobic amino acid (Karpusas et al., 1997). The masking effect of carbohydrates led to a more thermodynamically favorable form of IFN β that was less prone to aggregation.

Glycosylation also affects *in vivo* circulatory half-life time. Specifically, the number of terminal sialic acid capping the N-linked glycans was found to be heavily correlated to the clearance rate of glycoproteins. This property is extremely important for therapeutic application of glycoprotein and Section 2.2 will be devoted to this discussion.

2.1.2 Glycosylation and glycoforms

Most therapeutic recombinant proteins are made by eukaryotic cells. Heterogeneity in the glycoforms of proteins is a function of the types of organism used as production host. A better understanding of the origins of glycoforms and how to control them are becoming more important because the activity and efficacy of therapeutic recombinant proteins strongly correlates to the structure of the glycoprotein. In addition, from the policy perspective, the Food and Drug Administration (FDA) in the United States and Committee for Proprietary Medical Productions (CPMP) in Europe have demanded a better characterization of the carbohydrate structure of therapeutic glycoproteins for human therapy (Liu, 1992).

Glycoforms exist due to various factors. The first factor is the amino acid sequence of the glycoprotein itself. Oligosaccharide groups are bound to proteins via an N-glycosidic bond to the R-group of the Asn residue within the consensus tripeptide sequence Asn-X-Ser/Thr (where X is any amino acid but proline) and/or via an O-glycosidic bond to the R-group of Ser or Thr. The previous linkage is called N-linked glycans while the latter is called O-linked glycans (Figure 2.1 and 2.2). Glycans could also be attached as a component of the glycosyl phosphatidylinositol (GPI) membrane anchor. In the discussion of mammalian cell therapeutic protein glycosylation, GPI anchor linkage is ignored because no secreted glycoprotein has this form of oligosaccharide modification (Butler, 2004). Without the existence of this consensus peptide sequence, no carbohydrates will be naturally linked to decorate the backbone of a peptide. It was worth noting that there was an exception on the required consensus tripeptide for glycosylation. For example, N-linked glycosylation of protein C occurs at the consensus sequence Asn-X-Cys (Miletich and Broze, 1990). The existence of the consensus peptide sequence itself does not guarantee

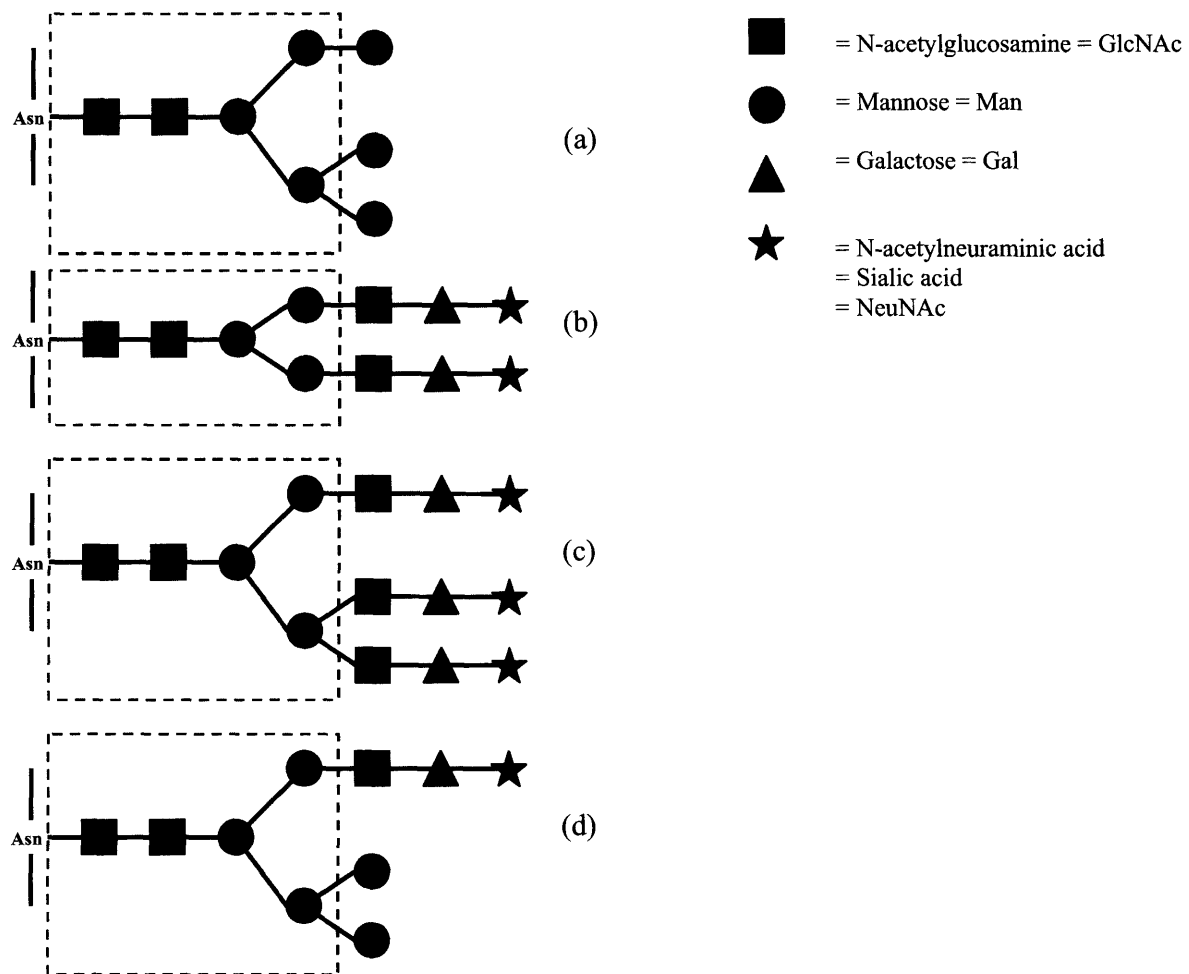


Figure 2.1 Structures of N-linked glycoprotein

There are three types of N-linked glycans: high mannose type (a), complex type (b,c), and hybrid type (d). The core structure of all types of N-linked glycans (which consists of two GlcNAc and three Man) is the same and the outer branches are the source of microheterogeneity. High mannose type contains additional mannose residues to decorate the core structure. Complex type contains two or more outer branches containing GlcNAc, Gal, and NeuNAc. Glycans with two branches are called bi-antennary (b) while those with three branches are called tri-antennary (c). Combinations of complex and high-mannose type are called hybrid type (d). These figures were inspired by Lodish et al. (1999).

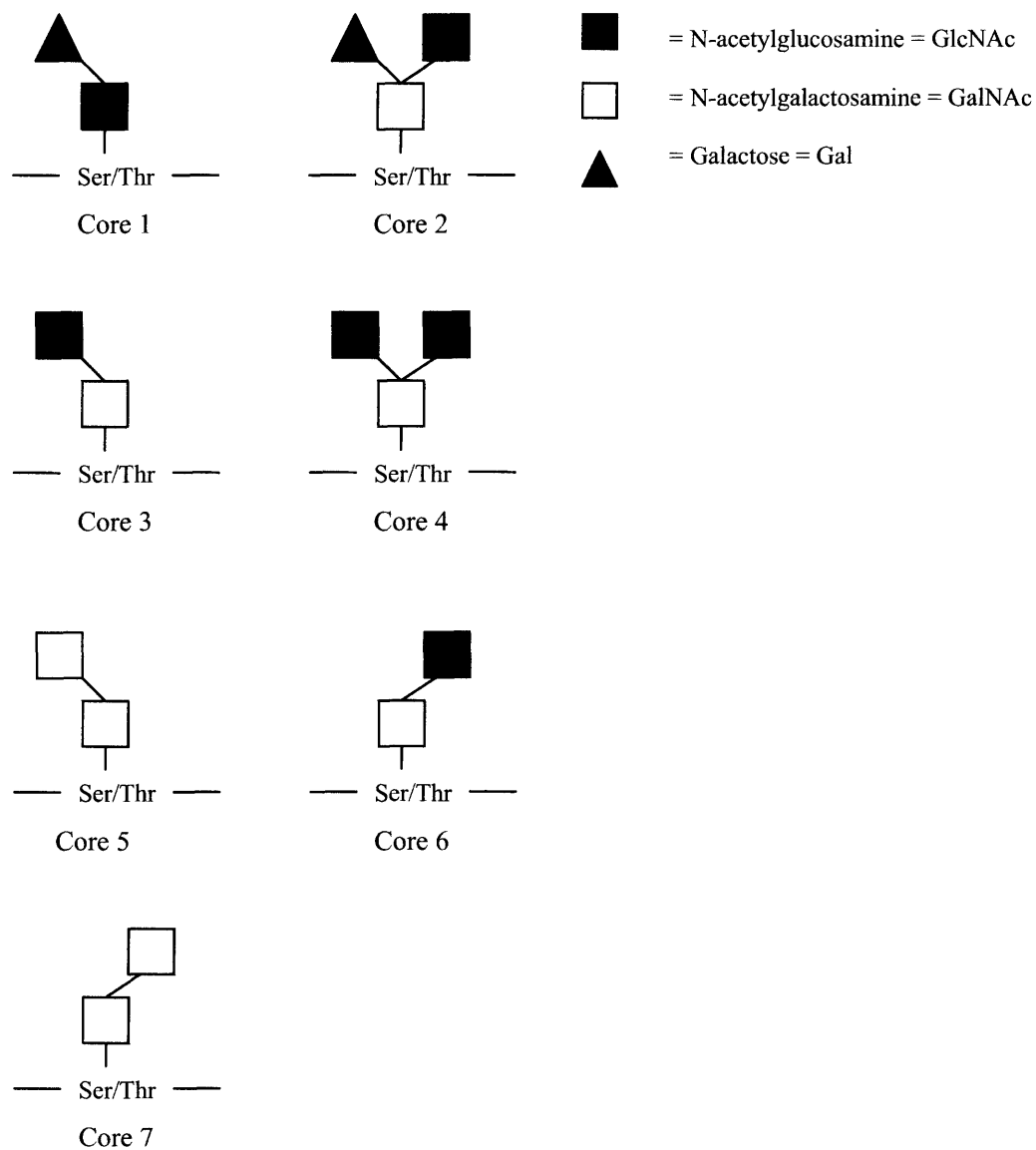


Figure 2.2 Core structures of O-linked glycosylation

There are at least 7 core structures of O-linked glycosylation. Core 1, 2, 3, 4 are more common than core 5, 6, 7. Further biosynthesis can produce fucosylated and sialylated lactosamines. These figures were inspired by Varki (2002).

glycosylation because other factor such as the tertiary structure of protein could contribute to the variable site occupancy. Asparagine located in the exposed region such as β -turn increases likelihood of glycosylation (Marshall, 1972). In fact, it was found by Gavel and co-workers that about 10% of the 465 of sites with the consensus sequence Asn-X-Ser/Thr were not glycosylated (Gavel and Heijne, 1990). Heterogeneity in glycoforms contributed to the fact that a peptide consensus sequence does not always translate to glycosylation are called *macroheterogeneity* (Figure 2.3).

The second factor contributing to the existence of various glycoforms was the availabilities and activities of the sugar precursors and enzymes related to glycosylation process that occurs in endoplasmic reticulum (ER) and compartments of the Golgi apparatus (reviewed in Kornfeld and Kornfeld, 1985, Goochee et al., 1991). It is worth noting that glycans attached on the sequon are not direct result of gene expression. Instead, they are a chain of enzyme-catalyzed monosaccharide addition reactions followed by some trimming reactions. The enzymes involved in the glycosylation process could be distinguished either as glycosyltransferase enzymes (which are primarily responsible for attaching a sugar chain to another) and glycosidase enzymes (which cleave a particular type of sugar). To understand what enzymes and sugar precursors are involved in the glycosylation, a review on the assembly of N-linked glycosylation is presented as follows.

First, precursor oligosaccharides are synthesized in the endoplasmic reticulum via addition of sugar monomers in step-wise manner. This precursor consists of lipid (dolichol) linked by a pyrophosphate bond to a glycan. During the synthesis of the precursor, the first two N-

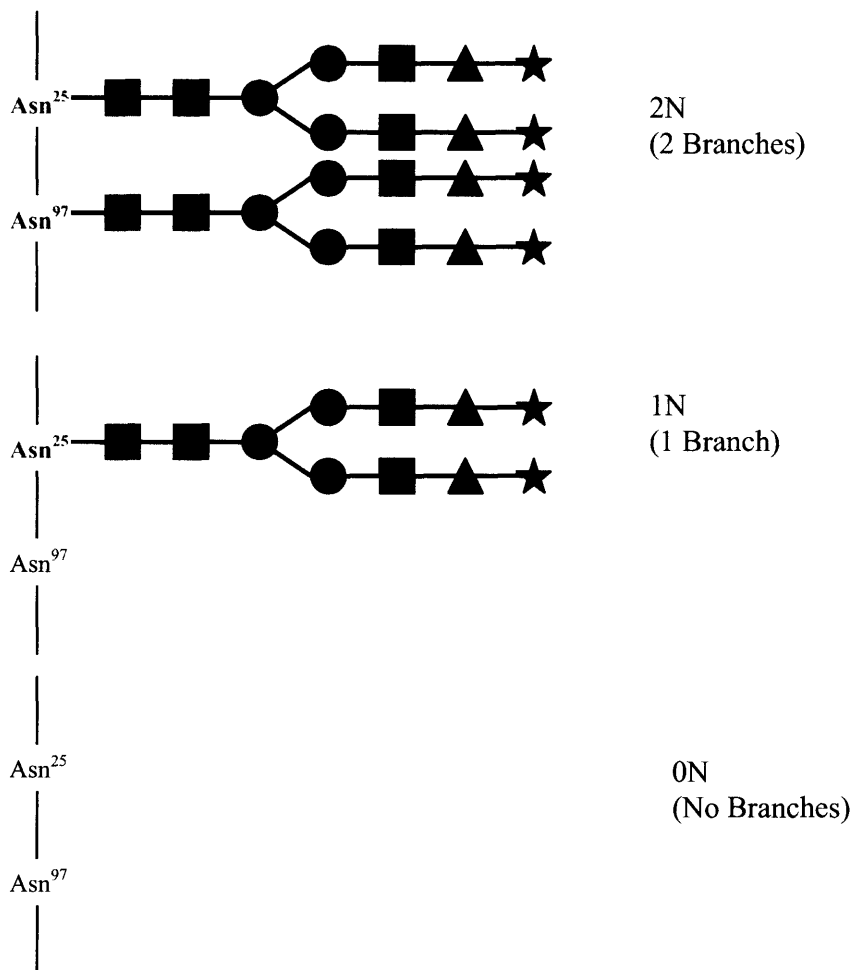


Figure 2.3 Macroheterogeneity of Interferon-γ glycosylation

Interferon-γ is used to illustrate macroheterogeneity in glycosylation. There are two potential site of N-linked glycosylation in Interferon-γ, Asn-25 and Asn-97. Therefore, there are three combinations of linkage: two oligosaccharide branches at both Asn-25 and Asn-97, one oligosaccharide branches at either Asn-25 or Asn-97, or no branches in either potential site.

acetylglucosamine (GlcNAc) molecules and the next five mannose (Man) molecules are obtained from UDP-GlcNAc and GDP-Man. Then, the next four mannose (Man) molecules and three glucose (Glc) molecules are obtained from the lipid intermediates Dol-P-Man and Dol-P-Glc. The final structure of the precursor is Glc₃Man₉GlcNAc₂-P-P-Dol. The second process in the assembly of N-linked oligosaccharides is the transfer of this precursor to the protein mediated by oligosaccharyltransferase (by recognizing the consensus peptide sequence) followed by an initial trimming step using α -glucosidase I, α -glucosidase II and ER α (1,2) mannosidase (Dempski and Imperiali, 2002). Glycosidase activities in cleaving glucose and α 1,2 mannose out of the precursor are quality control by the cell to ensure proper protein folding before the final modified precursor is sent to Golgi apparatus (Ellgaard and Helenius, 2001). The third process of the assembly of N-linked oligosaccharides is the transport of the trimmed precursors to the Golgi apparatus for more enzyme catalyzed sugar additions and trimming reactions. The whole process is summarized as Figure 2.4 and the enzymes involved are listed in Table 2.1.

Due to the involvement of various enzymes in glycosylation, glycoforms can arise due to competitive activities of the enzymes for the same substrate. The availability of sugar nucleotide donors is also another factor that contributes to the heterogeneity of glycoproteins. In addition, for proteins that contain many potential glycosylation sites, each site must compete for the same pool of enzymes. Heterogeneity in the components of oligosaccharides attached in the potential glycosylation sites as the result of these cellular factors is called *microheterogeneity* (Figure 2.1).

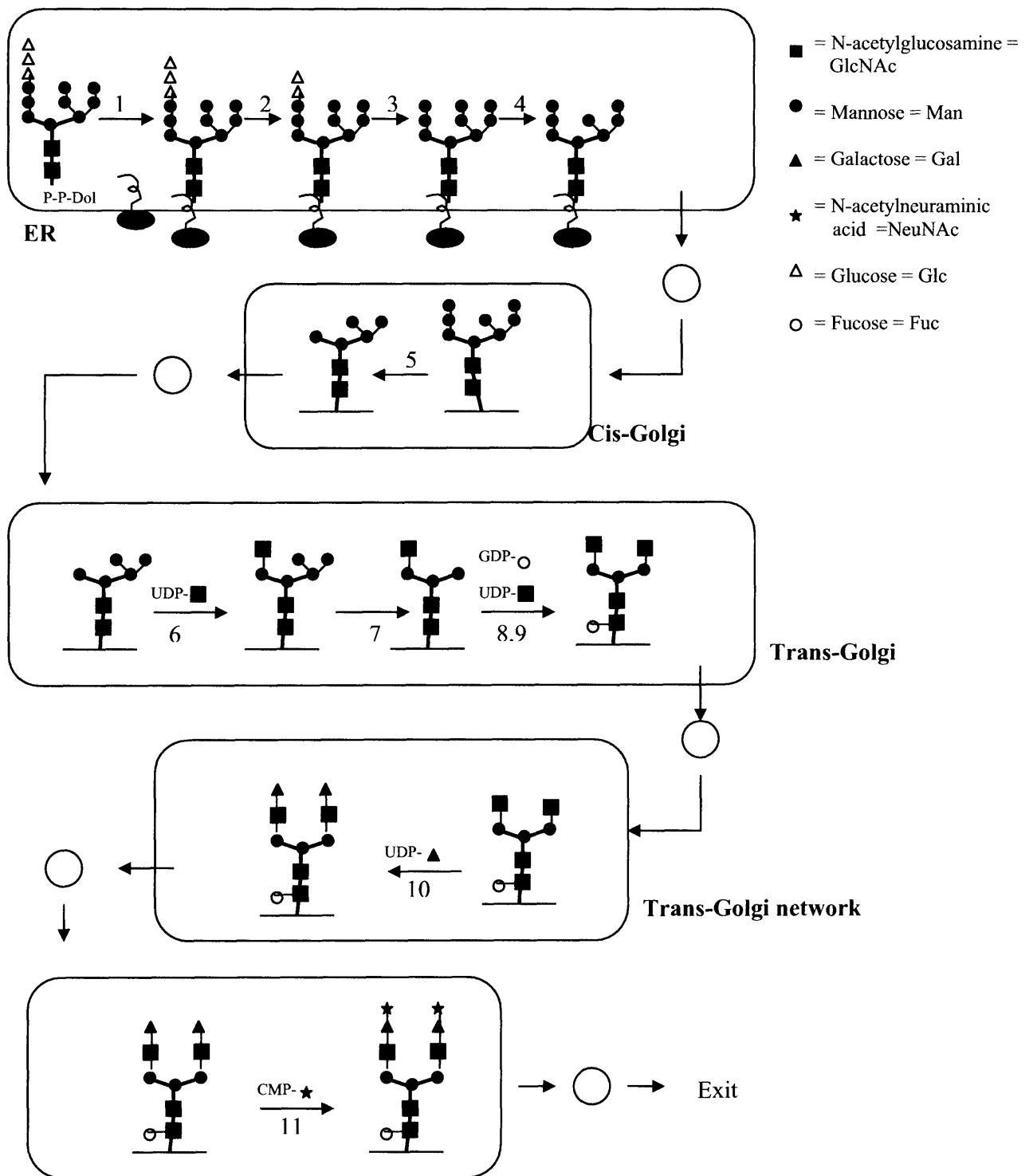


Figure 2.4 Assembly of N-linked glycans in mammalian cells

This figure is derived from review articles written by Kornfeld and Kornfeld (1985) and Goochee et al. (1991). Enzymes involved are listed on the next page.

Table 2.1 Enzymes involved in the assembly of N-linked oligosaccharides

ID in Figure 2.4	Enzymes
1	Oligosaccharyltransferase
2	α -glucosidase I
3	α -glucosidase II
4	ER α (1,2) mannosidase
5	Golgi α -mannosidase I
6	N-acetylglucosaminyltransferase I
7	Golgi α -mannosidase II
8	N-acetylglucosaminyltransferase II
9	α (1,6) fucosyltransferase
10	β (1,4) galactosyltransferase
11	α (2,3) sialyltransferase

2.1.3 Choice of host cells for glycoproteins

Heterogeneity of glycoprotein, either macroheterogeneity or microheterogeneity, is affected by the choice of host cells used to produce the glycoprotein. The fact that different cell types dictate glycoform patterns is shown to be related to the presence, concentration, kinetic characteristics, and compartmentalization of enzymes responsible for adding and/or cleaving sugars (reviewed in Rademacher et al., 1988). Therapeutic proteins that require glycosylation for biological activity are primarily produced using mammalian cells because these cells possess the cellular machinery and enzymes required to perform glycosylation to produce glycoprotein with glycoforms that resemble human protein. However, due to the complex media requirements (that translates to higher material cost) and slow doubling time, mammalian cell culture is found to be a very expensive and lengthy process. Therefore, much work has been done in other cell lines to produce glycoproteins with the correct oligosaccharides attached to the correct sequences and confirmations. In this section, a brief review is presented on the recent progress in engineering host cells to produce glycoprotein with correct glycoforms.

E. coli, despite its high growth rate and inexpensive medium required for protein production, is not the preferred host for glycoprotein production. The main reason why bacterial host is not used to produce glycoprotein is that the such an organism does not have the cellular components needed to attach carbohydrates to the backbone of peptides (Stanley, 1992). For example, endoplasmic reticulum and Golgi apparatus, which are needed for the first and second step of N-linked oligosaccharides assembly, do not even exist in *E.coli*. Recently, it has been demonstrated that food-borne pathogen *Campylobacter jejuni* has pgl gene cluster that is very similar to Stt3, a protein essential for oligosaccharyltransferase (Wacker et al., 2002; Feldman et al., 2005).

Overexpressing *C. jejuni* glycosylation machinery in *E.coli* produced N-linked glycan attachments at the consensus tripeptide sequences similar to mammalian cells (Asn-X-Thr/Ser). Unfortunately, the structure of the oligosaccharides, which consists of 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH), hexose, and five N-acetylhexosamine, does not resemble the structure of glycans produced by mammalian cells machinery (Wacker et al., 2002). Therefore, up to now, *E.coli* application to biopharmaceutical production has been limited to production of proteins that does not require the existence of oligosaccharides for bioactivity, such as hG-CSF (Jevsevar et al., 2005).

Besides *E.coli*, yeast has also gained popularity in producing therapeutic proteins that do not require glycosylation. Insulin, industrial enzymes, granulocyte-macrophage colony stimulating factor, and hepatitis B surface antigen are examples of products in the market expressed by *Saccharomyces cerevisiae* (Gerngross, 2004). Unlike *E.coli*, yeast possesses cellular components and a number of enzymes require for glycosylation. In fact, the first step of the N-linked glycan assembly in the ER of yeast produces Man₈GlcNAc₂-containing glycoprotein, which is similar to product from ER of mammalian cells (reviewed in Wildt and Gerngross, 2005). This Man₈GlcNAc₂-containing glycoprotein is then transferred to Golgi apparatus for further processing where the path between yeast and mammalian cell start to diverge due to different sets of enzymes. In mammalian cell culture, Man₈GlcNAc₂-containing glycoprotein is trimmed by mannosidases and modified by GlcNAc transferases before being processed further in medial-Golgi and trans-Golgi (Goochee et al., 1991). This is not true for yeast because Golgi apparatus of *S. cerevisiae* contains α -1,2-, α -1,3, and α -1,6 mannosyltransferase in addition to mannosylphosphate transferases (Gemmell and Trimble, 1999). As a result, N-glycan structures

from yeast tend to be mannosylated and hypermannosylated. In *Candida albicans*, the mannose content in N-linked glycoprotein could be as high as 200 mannose units and this hypermannosylated glycoprotein is hypothesized to interfere with the biological activity and to be immunogenic for therapeutic applications (Cutler, 2001; Brooks, 2004).

A genetic manipulation has been done extensively on *Pichia pastoris* to produce complex N-glycans similar to human cells (Choi et al., 2003; Hamilton et al., 2003; Bobrowicz et al., 2004). In fact, the asialylated form of human-like biantennary N-glycans has been successfully produced by eliminating mannosyltransferase and correctly localizing mannosidases I, II, GlcNAc transferases I,II, UDP-GlcNAc transporter, and fusion protein consisting of UDP-glucose 4-epimerase and β -1,4-galactosyl transferase (Bobrowicz et al., 2004). The biggest challenge remained in the yeast culture is to cap the N-glycans produced by this engineered *P. pastoris* with sialic acid. Without sialic acid as penultimate sugar, glycoproteins will be cleared out immediately from the body through the receptors in the liver (Ashwell and Hartford, 1982). Unfortunately, yeast does not have the source of endogenous sialic acid and sialyltransferase. In addition, various genes required for the synthesis of CMP-sialic acid do not exist in yeast (Gerngross, 2004). Further work to genetically introduce sialic acid as penultimate sugar must be done before yeast can replace mammalian cells as the preferred host to produce therapeutic glycoprotein.

In addition to therapeutic protein production in mammalian cells, *E.coli*, and yeast, there have been some advances in engineering plants, insect cells, and transgenic animals to produce glycosylated proteins. Plants are considered to be attractive because unlike bacterial host, plants

could correctly produce multimeric proteins and unlike animal cells, plants are safer hosts since plants cannot function as the hosts of pathogens such as prions and viruses (reviewed in Larrick and Thomas, 2001). In the assembly of N-linked glycans, plants can synthesize glycan $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$, which is similar to what is assembled in the trans-golgi of mammalian cells (Figure 2.4). However, the subsequent glycosylation steps diverged from mammalian cells. Instead of having α -1,6-linked core fucose, galactose, and sialic acid containing N-glycans, plant cells attach α -1,3-linked core fucose and β 1,2-xylose (Lereouge et al., 1998, Bakker et al., 2001). To attach galactose and sialic acid on glycoprotein produced by transgenic tobacco, Misaki and his co-workers overexpressed β 1,4-galactosyltransferase and performed *in vitro* sialic acid transfer (Misaki et al., 2003). Nevertheless, over 30% of the product still contained xylose and the structures of the glycoforms were truncated on one antenna. This is undesirable because the presences of 1,3-fucose and xylose in glycoproteins have been linked to immunogenicity and allergenicity (Van Ree et al., 2000). Recent work by Strasser et al. (2004) demonstrated that knocking out β 1,2-xylosyltransferase and α 1,3 fucosyltransferase could successfully remove immunogenic and allergenic sugars out of the N-linked glycans. However, it was observed that the one branch of N-linked glycans was not galactosylated and sialylated. A combination of Strasser et al. (2004) and Misaki et al. (2003) technique could potentially produce a completely sialylated glycoprotein without immunogenic and allergenic glycans.

Glycoproteins produced in insect cells shared the common problems faced by other non-mammalian host cells (reviewed in Tomiya et al., 2004). Insect cells are not able produce sialylated glycoproteins. In fact, most N-linked glycans produced by insect cells are in the form of paucimannosidic glycans and/or oligomannose glycans. In addition, like plants, insect cells

fucoylate the N-linked glycans in the $\alpha(1,3)$ position, which renders the glycoprotein allergenic (Wilson et al., 1998). Incorrect N-glycans formed in insect cells are caused by the lack of crucial transferases (such as galactosyltransferase and sialyltransferase) and the presence of undesired glycosidases (acetylglucosaminidase). β -1,4-galactosyltransferase was found in Tn-5B-1-4 cells at 10% the activity level as in Chinese Hamster Ovary (CHO) cells while Sf9 cells did not seem to have significant level of galactosyltransferase (Abdul-Rahman et al., 2002). Sialyltransferase activity was recently found in the embryonic stage of *D. melanogaster* central nervous system, suggesting that glycosylation occurs in cell-specific and developmental stage-specific manner (Koles et al., 2004). The existence of β -N-acetylglucosaminidase in many insect cell lines (such as Sf21, Bm-N, and Mb0503 cells) to cleave GlcNAc linkage prevents the elongation of N-glycans (Altmann et al., 1995). Recently, genetic engineering of Sf9 cells was successful in producing humanized sialylated glycoprotein (Aumiller et al., 2003). Two enzymes of CMP-sialic acid biosynthetic pathways (Nacetylneuraminate-9-phosphate synthase and CMP-sialic acid synthase) along with five mammalian glycosyltransferases (GlcNAcT I, GlcNAcT II, b4GalT, ST3Gal and ST6Gal) were expressed in Sf9 cells. Nevertheless, to achieve sialylation, serum supplementation or N-acetylmannosamine supplementation was necessary.

Transgenic animals have been extensively studied since the pioneering transgenic mice studies in 1987 (reviewed in Houdebine 2000; Dyck et al., 2003). Numerous proteins, such as β -lactoglobulin, human tissue plasminogen activator (tPA), interferon α -2b, granulocyte macrophage-colony stimulating factor (hGM-CSF), EPO, thrombopoietin have been produced from animal's serum, urine, seminal plasma, egg white, silk worm cocoon, and milk (Gordon et al., 1997; Simons et al., 1997; Ryoo et al., 2001; Rapp et al., 2003). For a production scale of 50

kg/year, cost-of-goods (including capital, production, and purification cost) of utilizing transgenic animals are estimated to be \$200/gram cheaper than utilizing cell culture (Dyck et al., 2003). In addition to the slightly lower cost-of-goods, transgenic animals also possess complex post-translational modifications necessary to produce glycosylated proteins. However, there are a few factors limiting transgenic animals use. Transgenesis efficiency is very low accompanied by hurdles in purification (Wilkins et al., 1992; Hofmann et al., 2003). Proteins that naturally occur in the milk or egg can easily be secreted at high rate but proteins that do not exist naturally in a particular system (such as an attempt to produce human factor VIII in milk) are not easily expressed (Devinoy et al., 1994; Paleyanda et al., 1997). Glycosylation in transgenic animals were found to be variable. Antithrombin III from goat milk sialylation was lacking while human protein C produced from milk of mice was not fully active (reviewed in Houdebine, 2003). It was hypothesized that poor glycosylation originated from the inappropriate folding in the ER and the inaccessibility of the enzymes needed for glycosylation in the Golgi apparatus. Regulatory, ethical, and social issues could potentially hinder the progress of utilizing transgenic animals to produce therapeutic glycoproteins.

Considering the problems faced with *E.coli*, yeast, plants, insect cells, and transgenic animals host, production of glycosylated therapeutic protein is still best carried out in mammalian cell system. In 2004, about 70% of the recombinant biopharmaceuticals were produced by mammalian cells (Wurm, 2004). Mammalian cells have a complex nutrient requirement in order to survive in the *in vitro* cell culture (Xie and Wang, 1994a). This translates to a higher cost incurred in the protein production utilizing mammalian cells as heterologous glycoprotein host. However, this high production cost is compensated with the secretion of biologically active

glycoproteins with terminal sialic acid acting as a biological mask. The importance of sialic acid will be elucidated in Section 2.2. Hamster cells (such as BHK and CHO) and mouse cells (hybridoma, myeloma, C127, J558L) are the commonly used mammalian cells to produce glycosylated proteins (reviewed in Jenkins et al., 1996; Walsh, 2003). One of the glycoforms produced by mouse cells is the α -linked-galactose residue (Gal α 1,3-Gal β 1,4-GlcNAc) which does not exist in human and is immunogenic (Sheeley et al., 1997; Baker et al., 2001). Mouse cells also tend to sialylate the glycoproteins with N-glycolylneuraminic acid (NeuGc), a derivative of sialic acid (NeuAc) that is immunogenic (Baker et al., 2001). Hamster cells, on the other hand, do not share the immunogenic properties due to α -linked-galactose residue and NeuGc cap because hamster cells do not actively express α -1,3 galactosyltransferase (which is the enzyme responsible to produce Gal α 1,3-Gal β 1,4-GlcNAc immunogenic residues) and make much greater amounts of NeuAc than NeuGc (Smith et al., 1990; Hokke et al., 1995). However, hamster cells do not express α 2,6-sialyltransferase and as a result, only produce α 2,3 terminal sialic acid utilizing α 2,3-sialyltransferase (Lee et al., 1989). Glycoproteins in human cells, on the other hand, are capped either with α 2,3 terminal sialic acid or α 2,6 terminal sialic acid. To establish the presence of α 2,6 terminal sialic acid in glycoproteins produced by hamster cells, many researchers successfully transfected hamster cells with α 2,6-sialyltransferase (Zhang et al., 1998; Bragonzi et al., 2000). During *in vivo* pharmacokinetics study of IFN γ produced by CHO cells, IFN γ with over 40% sialic acid in α 2,6 linkage took longer to clear from the blood compared to the IFN γ with only α 2,3 terminal sialic acid (Bragonzi et al., 2000).

In this subsection, a brief review on how different choices of expression systems lead to different glycoforms is presented. Bacteria do not possess the machinery for proper glycosylation. On the

other hand, other non-mammalian system produces non-sialylated forms of glycoproteins that do not resemble human glycoprotein: yeast produces hypermannosylated glycoprotein, plants produces xylosylated glycoprotein, while insect cells produces paucimannosidic glycans and/or oligomannose glycans. Mammalian cells are still the best host to produce sialylated glycoproteins. N-glycan structures that are produced by the most recent engineered cell lines are shown in Table 2.3.

Table 2.2 Structure of N-glycans produced by various expression systems

Cell	Timeline		N-glycans		Structure		Source
	Historically	Currently	No glycans	No glycans like glycans	No glycans		
Bacteria (<i>E.coli</i>)		Currently			Asn – DATDH – (HexNAc) ₂ – HexNAc – (HexNAc) ₂		Wacker et al., 2002
Yeast	Historically		High mannose		$ \begin{array}{c} \\ \text{Asn} - (\text{GlcNAc})_2 - \text{Man} \\ \\ \text{Man} - \text{Man} \begin{array}{l} \diagup \text{Man} \\ \diagdown \text{Man} \end{array} \end{array} $		
	Currently		Complex non-sialylated		$ \begin{array}{c} \\ \text{Asn} - (\text{GlcNAc})_2 - \text{Man} \\ \\ \text{Man} - \text{GlcNAc} - \text{Gal} \end{array} $		Bobrowicz et al., 2004
Plants	Historically		Xylosylated, Fucosylated		$ \begin{array}{c} \\ \text{Asn} - \text{GlcNAc} - \text{GlcNAc} - \text{Man} \\ \quad \quad \\ \text{Fuc} \quad \text{Xyl} \quad \text{Man} \end{array} $		
	Currently		Truncated xylosylated, sialylated		$ \begin{array}{c} \\ \text{Asn} - \text{GlcNAc} - \text{GlcNAc} - \text{Man} \\ \quad \quad \\ \text{Xyl} \quad \text{Man} - \text{GlcNAc} - \text{Gal} - \text{NeuAc} \end{array} $		Misaki, 2003
Insect	Historically		Paucimannosidic		$ \begin{array}{c} \\ \text{Asn} - \text{GlcNAc} - \text{GlcNAc} - \text{Man} \\ \quad \quad \\ \text{Fuc} \quad \text{Man} \end{array} $		
	Currently		Complex, sialylated		$ \begin{array}{c} \\ \text{Asn} - \text{GlcNAc} - \text{GlcNAc} - \text{Man} \\ \quad \quad \\ \text{Fuc} \quad \text{Man} - \text{GlcNAc} - \text{Gal} - \text{NeuAc} \end{array} $		Aumiller et al., 2003
Mammalian	Historically		Complex, sialylated		$ \begin{array}{c} \\ \text{Asn} - \text{GlcNAc} - \text{GlcNAc} - \text{Man} \\ \quad \quad \\ \text{Fuc} \quad \text{Man} - \text{GlcNAc} - \text{Gal} - \text{NeuAc} \end{array} $		
	Currently		Complex, sialylated		No changes (different terminal sialic acid linkage if $\alpha 2,6$ sialyltransferase is expressed)		Zhang et al., 1998, Bragonzi et al., 2000

DATDH = 2,4-diacetamido-2,4,6-trideoxyhexose, Hex = hexose, HexNAc = N-acetylhexosamine, GlcNAc = N-acetylglucosamine, Man = mannose, Gal = Galactose, Fuc = fucose, Xyl = xylose, NeuAc = sialic acid

2.1.4 Factors affecting glycosylation in mammalian cell culture

Based on a structured kinetic glycosylation model developed by Shelikoff and his co-workers, it was predicted that N-linked glycosylation macroheterogeneity in cell culture is affected by six factors (Shelikoff et al., 1996). They are: oligosaccharyl dolichol availability, oligosaccharyltransferase activity, primary amino acid sequence within or close to sequon, total protein synthesis rate, translocation rate, and competition from other cotranslational events. Altering culture conditions with the intention to affect these six factors is likely to change macroheterogeneity of glycoproteins.

Dolichol phosphate supplementation was attempted to increase the availability of oligosaccharyl dolichol in the cell culture (Yuk, 2001). This hypothesis arose from the understanding that one important step in glycosylation is the upstream transfer of oligosaccharide from dolichol phosphate to a consensus peptide sequence. 60 – 180 µg/mL dolichol phosphate was introduced to IFN γ producing CHO cells, and it was observed that there was no improvement in the site occupancy. It was postulated that the failure in site occupancy improvement was contributed to the fact that the rate limiting step was the activity of enzymes in the dolichol pathway, rather than the amount of dolichol phosphate.

Sugar and sugar nucleotides are heavily involved in the biochemical reaction network of glycosylation. As a result, the availabilities of sugar and sugar nucleotides have been manipulated and mechanisms have been proposed to increase glycosylation. In a study done by Hayter and co-workers, it was observed that under glucose limitation, the proportion of non-glycosylated IFN γ increased from 12% to 19% as culture time increased (Hayter et al., 1992).

When glucose was fed as a pulse, the proportion of non-glycosylated declined, followed by rapid increase in glucose consumption, conferring the fact that the availability of glucose for cells affected IFN γ glycosylation. The same phenomenon was also observed by other researchers (Goldman et al., 1998; Nyberg et al., 1999). Nyberg et al. (1999) proposed that under glucose limitation, mammalian cells preferred to utilize carbon source for energy production. Consequently, nucleotide biosynthesis declined and with lower nucleotide triphosphate pool, glycosylation site occupancy was reduced.

Limiting the amount of glutamine also affected glycosylation. In the fed-batch study of IFN γ producing CHO cell cultures done by Nyberg et al., it was found that glutamine limitation reduced glycosylation by reducing amino sugar formation (Nyberg et al., 1999). When feeding strategy was altered to prevent extreme starvation, no changes in glycosylation profile was observed (Wong et al., 2005). Sialylation was also altered by performing glutamine limitation fed batch culture and this will be discussed further in Section 2.2.

Glucosamine and ammonia are known to be precursors in the UDP-GlcNAc synthesis pathway. By altering the amount of glucosamine and ammonia supplied in the cell culture, it was expected that glycosylation patterns would change. It was found that feeding more glucosamine or ammonia in the cell culture caused a decline in the glycosylation (Nyberg, 1998; Borys et al., 1994). It was also observed that sialylation decreased and glycan antennarity increased under higher glucosamine and ammonia (Andersen and Goochee, 1995; Gawlitzek et al., 1998). Increasing amounts of glucosamine and/or ammonia produced higher amount of intracellular UDP-GlcNAc. This would contribute to a more complex antennary glycans (Gawlitzek et al.,

1998). It was not quite clear, however, as to why increasing glucosamine and ammonia led to reduced glycosylation. Glucosamine itself could have a negative impact to glycosylation or the accumulation of UDP-GlcNAc depleted the supply of other precursors needed for glycosylation (Nyberg, 1998). Ammonia, on the other hand, could also affect glycosylation by a different mechanism such as by altering the intercellular pH. Since ammonia is a weak base, it is possible that accumulation of ammonia raised the pH of trans Golgi (Schneider et al., 1996). Changes in intracellular pH were known to affect glycosyltransferase activities (Gawlitze et al., 1998). As a result, a shift in pH due to higher ammonia concentration could reduce glycan site occupancy.

In addition to feeding sugars, sugar nucleotides, and precursors needed for glycosylation pathway, changes in glycosylation can also be induced by the environment of the cell culture (reviewed in Goochee and Monica, 1990; Andersen and Goochee, 1994). This includes culture pH, temperature, partial pressure of oxygen, partial pressure of carbon dioxide, and the addition of supplements such as growth factors, sodium butyrate, or lipid. In this section, the effect of culture environment on glycosylation will be emphasized while the effect of culture environment specifically on sialylation will be discussed in Section 2.2.

Serum supplementation in cell culture is frequently necessary to maintain healthy cell growth because of growth factors and important glycoproteins in the serum. It was also known that serum supplementation affected the glycosylation pattern. In monoclonal antibody IgG2b glycosylation study, it was shown that less truncated glycoproteins were produced by hybridoma CB.Hep-1 when serum content was increased from 1% to 8% (Cabrera et al., 2005). Greater percentages of truncated glycoproteins in serum free culture were not the result of cell lysis or

degradation of oligosaccharide side chain post secretion (Hayter et al., 1990, Robinson et al., 1994). Instead, viable cells secreted truncated and high mannose glycoproteins as culture time increased in serum free system. It is worth noting that for antibodies, galactosylation affects activity more than sialylation does (Boyd et al., 1995). It is not clear how serum supplementation increased glycosylation. This is complicated by the inconsistent content of serum from batch-to-batch.

Extracellular pH is another factor found to affect glycosylation. Initial studies by Rothman et al. demonstrated that altering extracellular pH affected glycosylation of IgG from hybridoma cells albeit insignificantly (Rothman et al., 1989). Another study by Borys et al. showed that glycosylation of mouse placental lactogen-I (mPL-I) decreases when culture pH was below 6.9 and above 8.2 (Borys et al., 1993). Optimal pH for maximal product secretion was not necessarily the same as optimal pH for glycosylation. IgG3 monoclonal antibodies produced by hybridoma R24 were found to be produced at four times less IgG production rate when the pH was shifted from optimal pH for maximal yield to optimal pH for producing more glycosylated antibodies (Muthing et al., 2003). Changes in glycosylation from altering extracellular pH were demonstrated, not due to degradation of glycans after secretion, rather it was due to intracellular pH changes which further leads to intracellular glycosylation changes.

Culture temperature effect on glycosylation has not been well understood. A study done by Hendrik and co-workers demonstrated that temperature has no effect on glycosylation of t-PA produced by batch CHO culture (Hendrik et al., 2001). Another study by Andersen et al. showed that three N-linked oligosaccharide of t-PA were more prevalent than two N-linked form at lower

temperature (Andersen et al., 2000), indicating a positive effect of lower temperature for higher glycosylation. Hypothermic culture could also negatively impact glycosylation. In a recent study by Fox, two N-linked glycans forms of IFN γ produced by CHO cells were found to decrease by 5-10% when bioreactor temperature was reduced from 37⁰C to 32⁰C (Fox, 2005). CHO cells cultivated in lower temperature exhibited growth arrest in G₀/G₁ phase (Kaufmann, 2001). This phenomenon could contribute to heterogeneity in site occupancy although other factors such as reduced translation elongation rate at lower temperature can not be ignored (Andersen et al., 2000).

In addition to modulating cell cycle by temperature alterations, sodium butyrate is another commonly used method. The addition of sodium butyrate has been known to increase the proportion of time in which cells remained in the G₁ phase (Hendrick et al. 2001). Although production of glycoproteins increased with the supplementation of butyrate, there were mixed results on the benefit of butyrate to increasing site occupancy (Andersen et al.; 2000, Mimura et al., 2001; Hendrick et al., 2001). In t-PA produced by CHO cells, Hendrick et al. (2001) reported no significant changes in glycosylation after the addition of 1 mM butyrate. On the other hand, a slight increase in glycosylation of t-PA was observed by Andersen et al. (2000). For recombinant antibodies IgG3, the production of less truncated oligosaccharides were observed as sodium butyrate dose was increased (Mimura, 2001). Glycosylation variation caused by sodium butyrate supplementation was possibly due to the same mechanism as of variation caused by lowering culture temperature since growth state dependency was observed in both scenarios.

Concentration of dissolved oxygen was also shown to affect glycosylation. Kunkel et al. (1998) showed that IgG1 produced by murine hybridoma CC9C10 had more core-fucosylated asialo agalacto glycans at dissolved oxygen (DO) below 50%. In tPA producing CHO cells, Lin et al. (1993) demonstrated no significant changes in glycosylation in mild or severe hypoxia. Sensitivity to changes in DO can be attributed by different metabolic patterns for different cell lines. As shown by Ogawa *et al.* (1992), hybridomas that were adapted to serum-free media possessed higher sensitivity to DO levels. Optimization of DO must be done on a cell-by-cell basis since energy metabolism is affected by changes in DO.

Lipid supplementation in batch culture of IFN γ -producing CHO was shown to improve glycosylation (Jenkins et al., 1994). Oligosaccharyltransferase enzymes activity modulation by lipid was one possible explanation for this phenomenon. It was also hypothesized that lipid supplements could modulate dolichol phosphate supply in cells. The latter hypothesis was very unlikely because supplementation of dolichol phosphate was demonstrated not to affect glycosylation significantly (Yuk, 2001).

2.2 Terminal Sialic Acid: Role, Synthesis, and Degradation

2.2.1 *Role of terminal sialic acid as biological mask*

Sialic acid is electronegatively charged acidic 9-carbon monosaccharide that contributes to the structural diversity of complex carbohydrates (Varki, 1992; Schauer, 1982). The term sialic acid is usually used to represent more than 50 known neuraminic acid derivatives (reviewed in Angata and Varki, 2002). The difference between each derivative is contributed by various substituents at the amino or hydroxyl groups (reviewed in Schauer, 2004). The most commonly found sialic acids in mammals are N-acetylneuraminic acid (NeuAc or Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc or NeuGc). In human, only NeuAc form of sialic acid is found because of mutations that have occurred during human evolution (Chou et al., 2002). Specifically, deficiency of NeuGc form was due to inactivation of the gene for CMP-N-acetylneuraminic acid hydroxylase (CMAH), which converts CMP-NeuAc into CMP-NeuGc in other mammals. The intravenous administration of molecules carrying NeuGc was known to elicit immune responses in human beings (Higashi, 1977). As a result, it is important to ensure only NeuAc form of sialic acid exists within glycoproteins for human therapeutic applications.

In glycoproteins, sialic acids are α -glycosidically linked to different positions of other sugars, most frequently to galactose or N-acetylgalactosamine and very rarely to N-acetylglucosamine or sialic acid itself (reviewed in Schauer, 1985; Angata and Varki, 2002). α -2,3 linkage to galactose, α -2,6 linkage to galactose or N-acetylgalactosamine, and α -2,8 linkage to another sialic acid are commonly seen in animals while others are found in echinoderms.

Terminal sialic acid has been known to serve as a biological mask. When terminal sialic acid was removed from the glycans, desialylated serum glycoproteins have significantly lower survival times in the circulation as compared to the sialylated counterparts (reviewed in Ashwell and Harford, 1982). Sialidase treatment of glycoproteins exposes the galactose residues of glycoproteins. Then, galactose-specific lectin on hepatocytes recognizes and binds the asialoglycoproteins for degradation. The specific mechanism of asialoglycoprotein clearance has been extensively studied by various researchers (reviewed in Ciechanover et al., 1983; Harford et al., 1984). Briefly, asialoglycoproteins are taken up by receptor-mediated endocytosis: ligand binds to receptors located on plasma membrane, and the complexes are internalized via coated pit. Due to the low pH at the endosome, the ligand-receptor complex is dissociated. Then, the ligand is transferred to lysosome to be degraded while the receptor molecules are recycled to the plasma membrane.

In addition to protecting glycoproteins from being recognized by receptors in the liver, sialic acid is also known to play a masking role in other systems (reviewed in Schauer et al., 1984). For example, the existence of sialic acid reduces the activities of cytotoxic complement-dependent factors against autologous, invasive human bladder tumor cells, IgG with the Fc receptor of human T lymphocytes, and many more (Schauer et al., 1984). The mechanism behind how sialic acid masks antigenic and receptor sites or how sialic acid prevents the degradative activities of protease and endoglycosidases on glycoproteins are not well-understood. It was thought that the negative charge of sialic acid had a role in covering the other part of the glycoprotein. Reduction of carboxyl residue in sialic acid to alcohol was found to significantly change the immunological properties of blood group substances and bacterial antigens (Jennings et al., 1984).

In vivo pharmacokinetics studies have been performed on numerous glycoproteins. For blood clotting Factor IX, removal of sialic acid resulted in the loss of clotting activities at the same time courses (Chavin and Weidner, 1984). For most other glycoproteins, the removal of sialic acid correlated with significantly faster serum clearance rates and lower biological activity. Changes in circulatory half life time after desialylation for selected glycoproteins are summarized in Table 2.4. It is worth noting that *in vitro* biological activity and *in vivo* biological activity post sialic acid removal most often are not correlated. Removal of sialic acid from human erythropoietin (EPO), hyperglycosylated recombinant human EPO (rHuEPO), and recombinant human thyrothropin (TSH) resulted in very low *in vivo* activity but increased *in vitro* activity (Fukuda et al., 1989, Spivak and Hogans, 1989, Szkudlinski et al., 1993). It was hypothesized this difference was due to hepatic clearance via asialoglycoprotein receptor *in vivo* which is in agreement to pioneering studies by Ashwell and Hartford (1982).

Further studies on purified individual isoforms of rHuEPO revealed that higher sialic acid content affected not only longer serum half life but also lower EPO receptor affinity (Egrie and Browne, 2001). This was in contrast to conventional wisdom that predicted a positive correlation between higher glycoproteins receptor affinity and more biologically active molecules. From this study, it was concluded that for EPO, serum clearance, instead of receptor binding affinity, is the key factor for *in vivo* activity.

Table 2.3 The effect of sialic acid removal on the circulatory half-life time of selected glycoproteins

Glycoproteins	Circulating half-life		References
	Sialylated	Desialylated	
Alpha-antitrypsin	5 days	5 min	Gan (1979), Perlmutter (1991)
Cholinesterases	17 hours	26 min	Saxena (1997)
Chorionic gonadotropin	1.5 hours	3 min	Batta (1979)
CTLA4Ig	14 hours	6 hours	Abrams (1999), Flesher (1995)
Erythropoietin (EPO)	3 hours	2 min	Egrie (2001), Fukuda (1989), Spivak (1989)
Factor VIII	4 hours	5 min	Sodetz (1977)
Gamma-glutamyltransferase	1.25 hours	25 min	Mortensen (1997)
Granulocyte colony-stimulating factor (G-CSF)	2.7 hours	18 min	Kato (1993), Watari (1997)
Luteinizing hormone (hLH)	1 hour	9 min	Burton (1996)

2.2.2 Sialidase and glycoprotein

Sialidase or neuraminidase (EC 3.2.1.18, N-acetylneuraminosyl glycohydrolase) is a member of the glycosidase families that catalyze the hydrolytic cleavage of sialic acid ketosidically linked to oligosaccharide chains of glycoconjugates (reviewed in Monti et al., 2002). There are at least four different types of sialidase found in mammalian cells. The first one is Neu-1 or lysosomal sialidase. Neu-1 has been cloned from human, mouse, and rats (Carrillo et al., 1997). Lysosomal sialidase was implicated in two lysosomal storage disorders: sialidosis and galactosialidosis (Chavas et al., 2005). The second type of sialidase is Neu-2 or cytosolic sialidase. Cytosolic sialidase was preferentially expressed in muscle tissues of rat and humans (Monti et al., 1999). Studies on rat indicated the possible involvement of cytosolic sialidase in the myoblast differentiation (Sato and Miyagi, 1996). Both Neu-1 and Neu-2 have possible involvements in cancer. Studies on murine B16 melanoma cells demonstrated suppression of pulmonary metastasis by overexpressing Neu-1 or Neu-2 (Tokuyama et al., 1997; Kato et al., 2001). The third and fourth type of sialidase are denoted as Neu-3 and Neu-4. Both Neu-3 and Neu-4 are membrane-associated enzymes. Numerous researchers suggested the possible involvement of membrane-associated sialidases in neuritogenesis and axonal growth and regeneration (Monti et al., 2002).

In mammalian cell culture, soluble cytosolic sialidase has been isolated and characterized from culture fluid of Chinese hamster ovary (CHO) cells (Warner et al., 1993). It was found that soluble cytosolic sialidase had optimal pH near 5.5 with retention of about 50% maximal activity at typical culture pH of 7.5 (Gramer and Gooch, 1993). Accumulation of cytosolic sialidase is a general phenomenon that is observed in many other industrial-relevant cell lines albeit with

different pH-activity profiles (Gramer and Goochee, 1994b). There were two potential mechanisms of sialidase release and accumulation in cell culture. The first one would be the release of soluble cytosolic sialidase during cell lysis. As demonstrated by Gramer and Goochee (1993), an increase of lactate dehydrogenase (LDH), which signified an increase in the cell lysis, correlated to proportional increase of extracellular sialidase activity in CHO perfusion cultures. The second possible mechanism of sialidase release was the secretion of lysosomal sialidase to the extracellular culture fluid. It was known that adding NH_4Cl would enhance the secretion of lysosomal sialidase. Based on this fact, a study was conducted and it was found that the contribution of lysosomal sialidase to extracellular sialidase activity in CHO culture was found to be less than 1% (Gramer et al., 1995). Therefore, removal of sialic acid from glycoprotein in supernatant must significantly be from the release of sialidase via cell lysis. Membrane-associated sialidase would not be contributing to extracellular sialic acid cleavage off glycoproteins because membrane-associated sialidase act only upon gangliosides and it did not have activity against sialylated glycoprotein (Ha et al., 2004).

2.2.3 Effect of cell culture conditions on sialylation of glycoproteins

Culture conditions can affect the content of sialic acid in glycoproteins by affecting two opposing sialylation processes. The first process is the intracellular additions of sialic acid by sialyltransferase actions, and the second process is the extracellular removal of sialic acid by sialidase action. There are many sugars, enzymes, and co-substrates involved in the metabolism of sialic acid (reviewed in Schauer, 2004). As shown in Figure 2.5, it is intuitive that cell culture conditions affecting any of these components could lead to an improvement or impairment in sialic acid content of glycoproteins.

The addition of sodium butyrate in cell culture has been linked to deterioration in the quality of sialylated glycoproteins. A study done by Sung et al. (2004) demonstrated that the addition of 3 mM butyrate in hTPO-producing CHO cells led to a reduction in α -2,3 sialic acid and a decrease in *in vivo* biological activity. In contrast, a study by Chotigeat et al. (1994) demonstrated that the addition of 1.5 mM butyrate in human follicle stimulating hormone (hFSH)-producing CHO cells led to an increase in sialic acid content possibly due to an increase in sialyltransferase activities. In each of the experiment, it was shown that the release of glycosidase was not modulated by butyrate additions. Santell et al. (1999) performed a metabolic labeling experiment and observed a dramatic reduction to near undetectable level of metabolically labeled sialic acid precursor once sodium butyrate was added to the culture. In the same study, it was shown that the reduction in sialic acid content of the protein was only from 6.1 to 5.0 mol sialic acid/mole of protein after 10 days in culture. It was hypothesized by Santell et al. (1999) that sodium butyrate might stimulate cellular endocytosis and the recycling of sialic acid derived from pre-existing sialylated glycoproteins released into the supernatant.

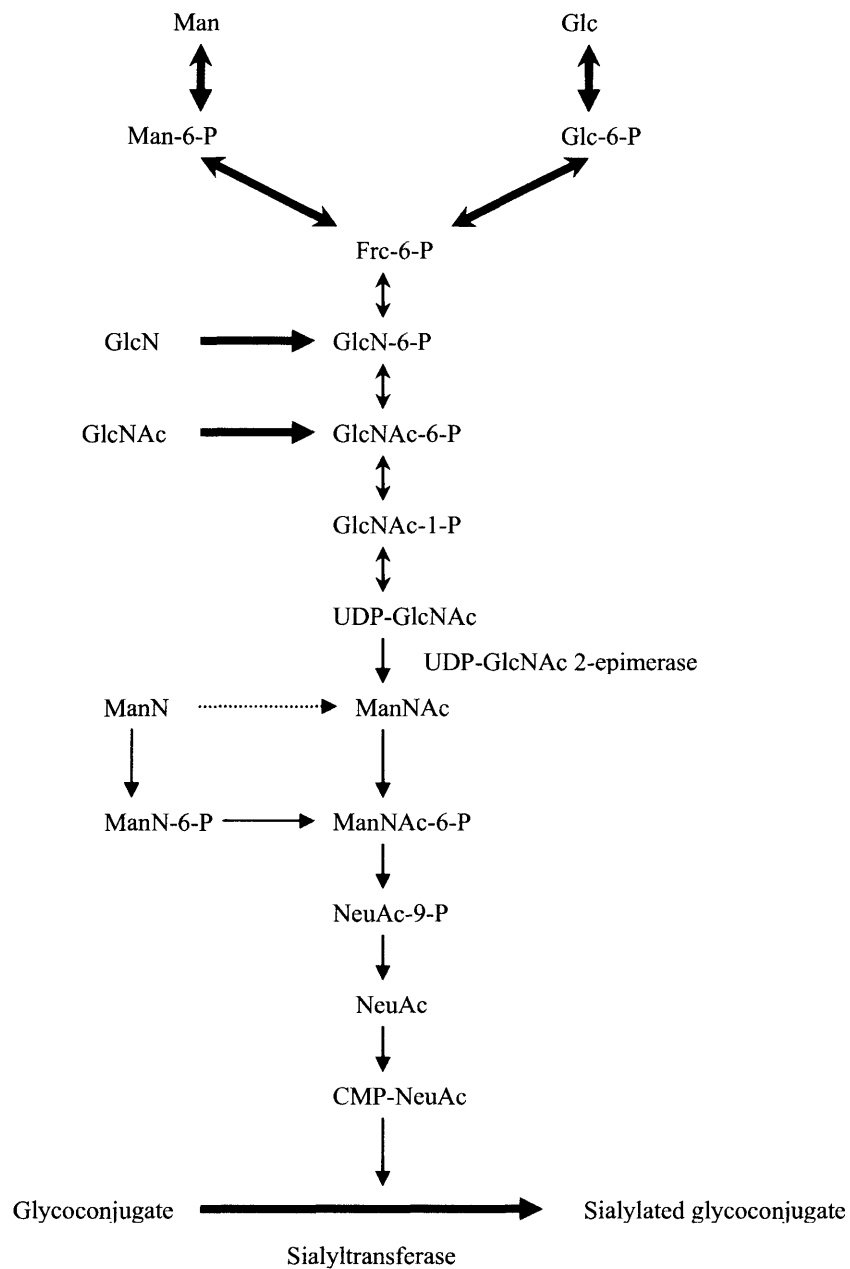


Figure 2.5 Schematic diagram of sialic acid metabolism

Sugars and enzymes involved in attachment of sialic acid to glycoconjugate are shown above based on the schematic diagram originally drawn by Keppler *et al.* (1999).

Different mode of operating bioreactors could lead to deterioration in sialylation. Watson et al. (1994) observed reduction in sialylated recombinant human tissue kallikrein (r-HuTK) produced by CHO cells when a microcarrier process was carried out in lieu of suspension cell culture. It was hypothesized that multiple layers of cells formed on the microcarriers. As a result, some cells might find limited access in obtaining media components needed for sialylation. Since no cell death data or glycosidase activities data was presented, it was not possible to eliminate extracellular degradation as one possible reason for lack of sialylated glycoproteins. Low-glutamine fed-batch strategy was found to impair sialic acid content in IFN γ produced by CHO cells as compared to typical batch culture (Wong et al., 2005). Since the cells were harvested in a high viability period, it was postulated that decrease in sialylation was due to impaired sialyltransferase activities or lack of nucleotide-sugar donors. Operating bioreactors in high dissolved oxygen could be beneficial for higher sialic acid content glycoprotein. In a study done by Chotigeat et al. (1994), recombinant human follicle stimulating hormone (hFSH)-producing CHO had an increased sialyltransferase specific activity from 1 to 4.9 nmol/mg/hour when the dissolved oxygen was increased from 10 to 90%. No detailed microheterogeneity analysis was conducted on the glycoprotein to draw a conclusion on sialylation profile of glycoproteins.

Serum supplementation in cell culture was found to affect sialylation pattern. Patel et al. (1992) demonstrated a reduction in sialic acid content of Ig-G1 produced by murine hybridoma from 6.9 nmol/mg to 3.4 nmol/mg when serum-containing media was used instead of serum-free media. A similar phenomenon was observed by LeFloch et al. (2004) during EPO production by α 2,6 sialyltransferase-expressing CHO cell line. Sialidase assays on culture medium supplemented by

serum showed insignificant level of endogenous serum sialidase activity. The reason why less sialic acid was obtained could be due to inconsistent complex content of serum or sialidase released by dead cells.

Elevated partial pressure of carbon dioxide ($p\text{CO}_2$) could alter protein sialylation. Increasing $p\text{CO}_2$ was found to detrimentally reduce poly sialic acid content in neural cell adhesion molecule (NCAM) produced by CHO cells (Zanghi et al., 1999). It was hypothesized that higher $p\text{CO}_2$ led to higher concentration of bicarbonate concentration and higher osmolality. In addition, higher $p\text{CO}_2$ would reduce culture pH and could potentially alter intracellular pH. Changes in intracellular pH may alter intraorganelle pH and affect sialyltransferase activity. The effect of elevated $p\text{CO}_2$ on sialylation was not observed on tPA-producing CHO cells (Kimura and Miller, 1997).

Altering culture temperature could affect sialic acid content of glycoproteins. As demonstrated by Yoon et al. (2003), during the death phase of cell culture, sialic acid content in EPO produced by CHO cells at 37°C dramatically declined from 11.8 to 6.9 mole sialic acid/mole EPO. On the other hand, when cell culture was performed at 30°C , reduction in sialic acid content was only from 12.7 to 10.2 mole sialic acid/mole EPO. The difference in sialic acid reduction during the death phase could be contributed to the weaker sialidase activity at lower temperature (Gramer and Goochee, 1993). This effect was not observed by Bollati-Fogolin et al. (2005) as temperature shift experiment was performed on human granulocyte macrophage colony stimulating factor (hGM-CSF)-producing CHO cell culture. It was not clear at what point of cell culture the hGM-CSF was collected for oligosaccharide structural characteristic analysis in this study. If the

glycoprotein in Bollati-Fogolin's study was collected during active growth phase, direct comparison between Bollati-Fogolin and Yoon studies can not be directly compared.

Supplementation of cell culture with glycerol had the potential to increase sialic acid content of glycoproteins. As demonstrated by Rodriguez et al. (2005), interferon- β produced by CHO cells has an increased sialic acid content from 2.3 to 2.9. Sialic acid content was defined here as the ratio of disialylated biantennary structure and monosialylated biantennary structure. It was also shown that glycerol supplementation has the benefit of reducing aggregation by more than 50%. The utilization of glycerol should be considered carefully as growth rate declined with increasing glycerol content.

N-acetylmannosamine (ManNAc) supplementation to cell culture had mixed results in CHO cultures. Gu and Wang (1998) demonstrated that supplementation of ManNAc up to 20 mM led to 15% improvement in sialic acid content. However, feeding 40 mM ManNAc was found to produce no further improvement in sialic acid content. In separate studies by Baker et al. (2001), it was shown that supplementation of ManNAc to NS0 and CHO cells increased interacellular content of CMP sialic acid. However, this did not translate to increased sialylated N-glycans of TIMP-1 glycoproteins. It was suggested that CMP-sialic acid transport limitation into the Golgi lumen could be a limiting factor or perhaps, CMP-sialic acid transport capacity was inhibited by UDP-HexNAc or CMP.

Addition of sialidase inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (2,3D) was an effective way to maintain sialic acid consistency throughout cell culture. In IFN γ -producing

CHO cultures, it was observed that 15% reduction in sialic acid content during death phase could be prevented by supplementing 1 mM 2,3D at the beginning and at 72 hours of cell culture (Gu et al., 1997). Similar results were demonstrated by Gramer et al. (1995) when 1 mM 2,3-D was added to the gp120-producing CHO cell culture. Adoption of this method for an industrial-relevant process was unacceptable due to the high cost incurred for large scale process (Ferrari et al., 1998).

2.2.4 Genetic engineering methods to improve sialylation

Genetic engineering can be used to create a robust cell line that produces improved sialylated glycoprotein from the beginning of the culture. This is in contrast to employing cell culture modifications which are very non-specific. For example, altering culture temperature could potentially reduce sialidase activity moderately but simultaneously impair cell growth rate. The basis behind genetic engineering methods is similar to the basis behind cell culture condition adjustments, which is either to affect sialic acid incorporation intracellularly or to affect sialidase degradative activities extracellularly.

Sialyltransferase, a critical enzyme involved in transferring sialic acid to glycoconjugate (Figure 2.5), has been overexpressed in CHO cells to improve sialic acid content of glycoproteins. Weikert et al. (1999) transfected human α 2,3-sialyltransferase to TNFR-Ig producing-CHO and observed an improvement of sialic acid from 4 to 6 mol sialic acid/mol protein. Attempt to overexpress α 2,6-sialyltransferase, which was a form of sialyltransferase naturally not occurring in CHO cells, have also been attempted by many researchers (Bragonzi et al., 2001; Fukuta et al., 2000). A slight improvement from 1.12 to 1.16 mole sialic acid/mole IFN γ protein was observed by Bragonzi et al., while improvement of sialylation extent from 61.2% to 79.8% in IFN γ produced by CHO was observed by Fukuta et al. Overexpressing both α 2,3 and α 2,6-sialyltransferase was done by Fukuta et al. (2000) and an improvement of 20-25% in extent of sialylation in IFN γ was obtained. In all these studies, no complete sialylation was achieved, implying the availability or activity of sialyltransferase is not a limiting factor.

Sialidase, a critical enzyme responsible for cleaving sialic acid during death phase of cell culture, has been knocked down utilizing an antisense method (Ferrari et al., 1998). Sixty percent of sialidase activity was reduced with improvement of 0.6 – 1.1 mole of sialic acid/mole DNase. Data originated from only one period of cell culture was compared to justify the success in sialic acid improvement. It was not obvious whether the sialic acid content was maintained high throughout the culture to substantiate the benefit from sialidase reduction.

2.3 RNA Interference Overview

2.3.1 Mechanism of RNA interference (RNAi)

A novel method to silence a gene is to introduce double stranded RNA (dsRNA) homologous in sequence to the desired target's DNA which further induces sequence specific post-transcriptional gene silencing in many organisms (reviewed in McManus and Sharp, 2002; Dykxhoorn et al., 2003; Novina and Sharp, 2004). Such silencing effects result from an antisense mechanism that depends on hybridization between the injected RNA and endogenous RNA messenger. This method, which is further known as RNA interference (RNAi), was widely known after the discovery that injection of dsRNA into the nematode *Caenorhabditis elegans* led to the specific gene silencing of genes homologous in sequence to the injected dsRNA (Fire et al., 1998). It was observed by Fire and his co-workers that the injection of double stranded RNA had a more pronounced silencing effect than injecting either the sense or the antisense strands alone. Furthermore, this silencing effect was carried out to F1 progeny of *C. elegans*.

At present, it is proposed that the mechanism of RNA interference consists of two distinct steps (Hutvagner and Zamore, 2002; Sharp and Zamore, 2000). The first one is the initiation step, which involves cellular uptake of long double stranded RNAs and the subsequent enzymatic digestion of these strands into 21 – 23 nucleotide (nt) of small interfering RNAs, which are often called guide RNAs (Hannon, 2002; Zamore et al., 2000). Parrish et al. (2000) concluded that any dsRNA segment greater than ~26 bp can generate RNAi. It is also observed that longer dsRNAs are more active than short dsRNAs (Sharp, 2001). One of the enzymes responsible in cleaving dsRNA into smaller pieces of siRNA has recently been identified as Dicer, which is a member of the RNase III family of nucleases (Bernstein et al., 2001, McManus and Sharp, 2002).

The second step of the RNA interference process is the effector step. In this step, the guide RNAs bind to RNA-induced silencing complex (RISC), a nuclease complex proposed to have helicase activity, endonuclease activity and homology searching activity (Hammond et al., 2001). Next, RISC complex unwinds the double stranded guide RNA and then the antisense strand of the guide RNA targets the homologous endogenous transcript via base pairing interactions. The endogenous transcript is cleaved by the RISC complex, resulting in the down regulation of the gene. The mechanism of RNA interference process is summarized in Figure 2.6.

Initially, RNA interference was thought to be ineffective for mammalian cells because the introduction of long double stranded RNA (approximately > 30 nucleotide) can trigger profound physiological response that lead to the activation of protein kinase PKR (Manche et al., 1992). Activated PKR, in turn phosphorylates and inactivates translation initiation factor, eIF2a, leading to the repression of translation. In other pathways, long dsRNAs activate RNase L, which further causes nonspecific RNA degradation (Minks et al., 1979). This problem can be solved by bypassing dsRNA and simply using siRNA. Elbashir et al. (2001a) showed that 21-nucleotide siRNA duplexes specifically suppress gene expression in human embryonic kidney and HeLa cells. The effectiveness of siRNA varies – with the most potent siRNA results in >90% reduction in target RNA and protein levels (Caplen et al., 2001, Holen et al., 2002). It was found that the design of siRNA matters, 21 nt dsRNAs with 2 nt 3' overhangs are most effective. Sequence specificity of siRNA is very stringent. Single base pair mismatches between siRNA and its target mRNA reduces the effectiveness of silencing severely (Elbashir et al., 2001a).

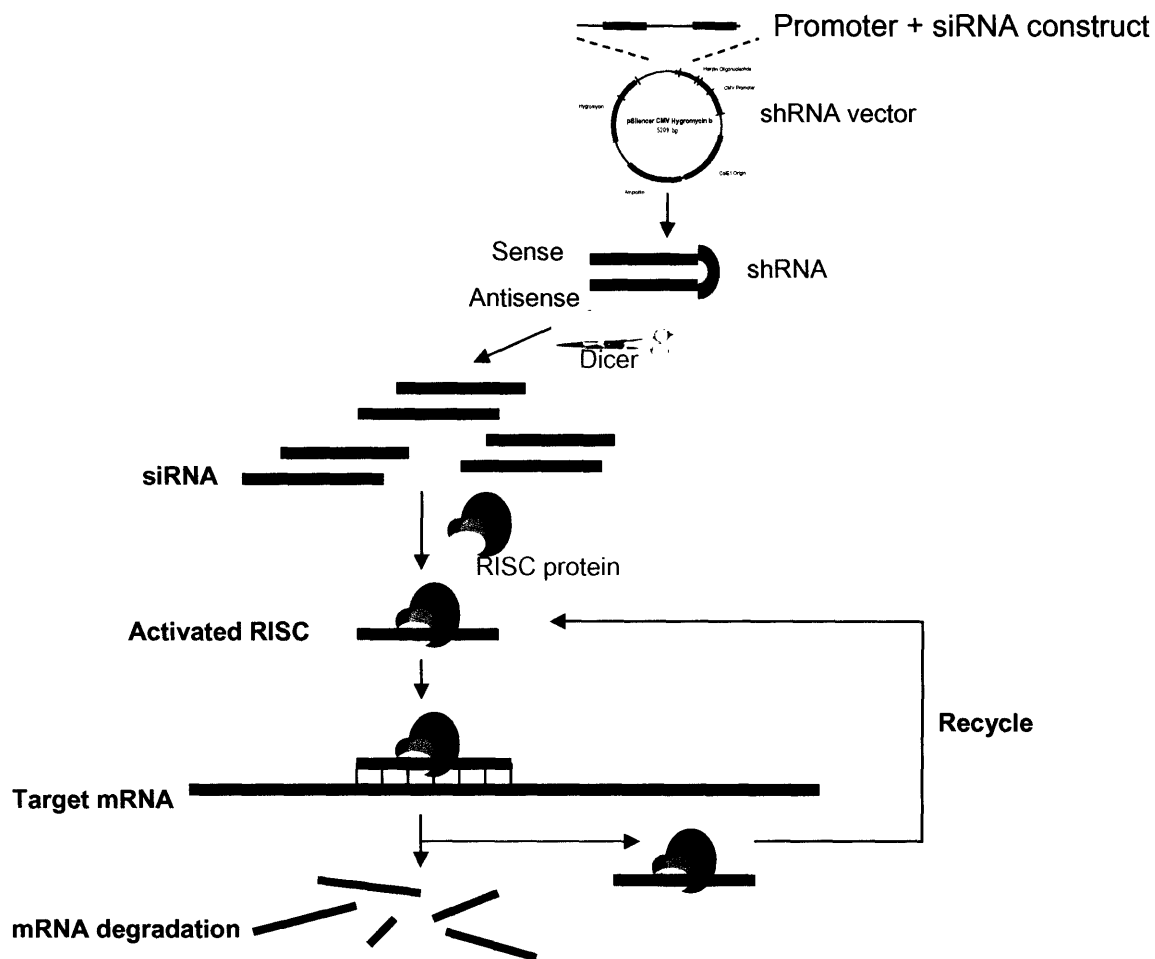


Figure 2.6 RNA Interference (RNAi) mechanism for gene-specific mRNA degradation

This figure represented RNAi mechanism utilizing siRNA derived from shRNA-producing vector. This illustration was inspired by earlier publications by Novina and Sharp (2004) and Dykxhoorn *et al.* (2003).

2.3.2 Transient and stable silencing by RNAi

In mammalian cell systems, RNAi processes can be induced by transfecting chemically or enzymatically synthesized double-stranded siRNA. This process is transient by nature because of dilution of chemically synthesized and transfected siRNA to the next generation or degradation of the siRNA. The duration of chemically synthesized and transfected siRNA in mammalian cells ranges from 24 – 120 hours (Caplen et al., 2001; Holen et al., 2002; Ohta et al., 2002). Re-transfection of chemically synthesized siRNA or establishing stable silencing system for long term silencing is required (reviewed in Dykxhoorn et al., 2003).

Stable silencing by RNAi mechanism in mammalian cells is typically accomplished by utilizing DNA-vector or virus-vector mediated RNAi. RNA polymerase III promoters (such as U6 and H1 promoters) are utilized to generate the formation of gene specific short hairpin RNAs (shRNA). These shRNAs are later processed by Dicer into siRNAs (Figure 2.6). RNA polymerase III promoters have the benefit of well-defined termination site, which consists of four or more T residues (Paule and White, 2000). U6 promoter, however, must have a guanosine in the +1 position, which restricts the choices of siRNA sequences that can be utilized for gene-specific silencing (Tuschl, 2000). H1 promoters, on the other hand, are more flexible because either adenosine, uridine, cytidine, or guanosine is permitted in the +1 position. RNA polymerase II promoters used to be incompatible for the mammalian cells application because these promoters produce long double stranded RNA which could trigger global non-specific inhibition of mRNA translation (Caplen et al., 2000). However, it has been demonstrated recently by Xia et al. (2002)

that modified RNA polymerase II promoters could induce gene-specific suppression in mammalian cells and mouse.

2.3.3 RNAi and glycoengineering of CHO cells

RNAi has been used as a tool to study various gene functions in CHO cells by suppressing gene-specific mRNA expression (Ohta et al.; 2002, Ma et al., 2003). Up to this day, only one publication demonstrated the application of RNAi in glycoengineering of CHO cells (Mori et al., 2004). Specifically, RNAi was used to knock down α 1,6 fucosyltransferase in CHO cells. It has been demonstrated by other researchers that defucosylated antibodies were desired because of the enhanced ADCC, which was an antibody effector function (Mori et al., 2004). Utilizing U6 promoter-driven siRNA expression system combined with a phenotypic selection strategy yielded two clones with 20-30% of parent's α 1,6 fucosyltransferase level. It was noted that the application of a stronger siRNA expression system could have been used to eliminate the need for a second selection step for identifying clones with reduced α 1,6 fucosyltransferase expression. Growth rate reduction was observed and this was postulated to be due to stress induced by puromycin selection.

2.4 Model System: Recombinant Human Interferon Gamma (IFN γ) Produced by Chinese Hamster Ovary Cells.

Chinese Hamster Ovary cell line is used as a model system because it is the most widely used mammalian cell for biopharmaceuticals production (Walsh, 2003). Human interferon gamma (IFN γ) produced by CHO cell is used a model glycoprotein because it has two potential sites for glycosylation: Asn-25 and Asn-97 (Nyberg, 1998). Interferon- γ is normally secreted by antigen-sensitized T-lymphocytes which stimulates the major histocompatibility complex (MHC) class II expression in cells present during immune response (Curling et al., 1990). IFN γ has potent antiviral activity and has been produced by various systems: *E.coli*, insect, yeast, and CHO cells (Bulleid et al., 1990). The presence of oligosaccharides on the backbone of IFN γ was shown to be unnecessary for the antiviral activity of IFN γ (Kelker et al., 1983). Despite this fact, IFN γ is still deemed to be an ideal system for the study because macroheterogeneity and microheterogeneity characterization method have been developed for precise characterization of IFN γ glycoprotein (Gu, 1997). In addition, it has been observed by Gu (1997) that sialic acid cleavage from glycoprotein during death phase of CHO cells was inevitable, making this system an ideal model for this thesis.

3. Materials and Methods

3.1 Cell Culture

3.1.1 Cell lines

There were two different Chinese Hamster Ovary (CHO) cell lines used in this thesis: CHO-IFN- γ and CHO-DG44. CHO-IFN- γ is a CHO cell line expressing IFN γ using an SV40 promoter. CHO-IFN- γ was created by cotransfecting DHFR⁻ CHO cell line with both DHFR and IFN γ genes. CHO-IFN γ was a kind gift from Dr. Walter Fiers many years ago and it has been used very extensively in our lab by previous researchers (Gu, 1997; Nyberg, 1998; Yuk, 2001; Fox, 2005). CHO-IFN- γ was grown in the presence of methotrexate, which is a competitive inhibitor of the DHFR enzyme. Methotrexate selection leads to gene amplification of DHFR and the adjacent gene, which increases the copy number of the therapeutic glycoprotein genes co-transfected with DHFR.

The second cell line used was CHO-DG44. CHO-DG44 was kindly provided by Prof. Lawrence Chasin (Columbia University, New York) and it is a double deletion mutant that contains no copies of the DHFR gene (Urlaub et al., 1986). CHO-DG44 is a popular cell line used to transfect a gene (for example, a gene encoding a recombinant therapeutic protein) to be produced in high copy number, as there have been many well-developed methods to introduce DNA efficiently (such as Ca₃PO₄ method or lipid based carrier method). In addition, subjecting the transfected cell line to increasing concentrations of methotrexate can easily allow for selection of stable cell lines bearing a high copy number of the gene of interests. During the cell culture,

CHO-DG44 required the supplementation of ribonucleosides because without DHFR genes, CHO cells are incapable of producing their own ribonucleosides.

3.1.2 Culture medium, transformation, and maintenance

The basal medium for all anchorage dependent Chinese Hamster Ovary cell culture discussed in this thesis was Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY). All supplements were obtained from Invitrogen and all chemicals were obtained from Sigma Aldrich (St Louis, MO) unless otherwise stated. Prior to 0.22 μm vacuum filtration sterilization, DMEM intended for CHO-IFN γ culture was supplemented with 0.25 μM methotrexate, 20 U/mL penicillin – 20 $\mu\text{g/mL}$ streptomycin mix, and 10% heat inactivated fetal bovine serum (IFS). For the culture of CHO-DG44 cell lines, alpha MEM supplemented with nucleosides and deoxynucleosides was used in lieu of DMEM. All supplements except methotrexate were the same as those formulated for CHO-IFN γ cell culture. In the absence of nucleosides and deoxynucleosides, DMEM can be used to grow CHO-DG44 with the supplementation of 16 μM thymidine and 0.1 mM hypoxanthine. These anchorage dependent CHO cells were grown in 6-well-plates, T-75 flasks, and T-150 flasks by inoculating about $1 \times 10^5/\text{mL}$ cells and were incubated at 37°C with 5 – 10% CO $_2$ overlay in a humidified incubator. The cells were passaged as necessary and the medium was replaced with fresh medium every 3-4 days.

Transformation from attached cell lines to suspension cell lines was performed by switching the medium from DMEM to HyQ PF-CHO (HyClone, Logan, UT) supplemented with 4 mM Glutamine and 0.1% Pluronic (Invitrogen). Initially, anchorage dependent CHO cells were grown in DMEM supplemented by reduced serum content (5% IFS instead of 10% IFS). When

the anchorage dependent cells with 5% IFS supplementation started to enter growth phase, these cells were transferred to suspension culture in which the medium was replaced with HyQ PF-CHO supplemented by 5% IFS. If the cells did not grow well, a mixture of 50% DMEM and 50% HyQ PF-CHO supplemented by 5% IFS could be used before the medium was switched entirely to HyQ PF-CHO supplemented by 5% IFS. Serum content was gradually reduced by diluting the growing cells with fresh serum-free HyQ PF-CHO. This way, serum content gradually was reduced by half at each dilution step (from 5% to 2.5% to 1.25% and so on). When the serum content was very close to zero and the cells showed a normal proliferation profile (indicated by doubling time of 24-48 hours), the medium was replaced entirely by serum-free HyQ PF-CHO. Transformation from anchorage dependent to serum free suspension cultures procedures could take place from 1 – 3 months.

Serum-free suspension CHO cultures were routinely maintained in sterile shake flasks agitated at 100 rpm on an orbital shaker located in 37°C incubator with a 5 – 10% carbon dioxide overlay. The most commonly used basal medium for suspension culture was HyQ PF-CHO supplemented by 4 mM L-glutamine, 0.25 µM methotrexate, a 20 U/mL penicillin – 20 µg/mL streptomycin mix, and 0.1% pluronic F-68. HyQ CDM-CHO could be used as basal medium although transformation time was relatively longer than when HyQ PF-CHO was used. Every 3-5 days, culture medium was replaced entirely with fresh medium and the cells were resuspended with a density of 2.5×10^5 cells/mL. All the experiments performed with 125-mL, 250-mL, 500-mL, and 1-L disposable Erlenmeyer flasks with a starting volume of 20% of maximal capacity of each flask. For example, experiments conducted in 1-L disposable Erlenmeyer flasks will be initiated by inoculating 2.5×10^5 cells/mL CHO in 200 mL fresh medium.

3.1.3 CHO Cell Bank Maintenance

Frozen stocks were prepared from cells collected at their growth phase when viability exceeded 95% by centrifuging these cells at 1000 rpm for 10 minutes. Freezing medium consisted of fresh DMEM or alpha MEM and 7% (v/v) dimethyl sulfoxide (DMSO) was prepared and placed in a refrigerator at 4°C for at least 30 minutes. A mixture of conditioned medium and fresh medium with 7% (v/v) DMSO should be used as freezing medium for suspension cell lines in which the serum content was gradually reduced to yield serum-free suspension cell lines. This freezing medium may be stored for up to one week at 4°C and up to a year at -20°C. 1×10^7 cells were diluted in 1 mL of chilled freezing medium and stored in 1.5-mL cryogenic vials. These vials were quickly transferred to Nalgene™ Cryo 1°C freezing container (which had been filled with 250 mL isopropanol) and placed into the bottom part of -80°C freezer for 12 hours. Using this container, the cells were frozen down slowly at the rate of cooling of -1°C/min to prevent massive cell death from sudden freezing. Within 12 – 24 hours, the cryogenic vials were transferred to a liquid nitrogen tank for long-term storage. The isopropanol must be disposed and replaced with the new isopropanol before the Nalgene™ Cryo 1°C freezing container can be re-used.

New cultures were initiated by removing the cryovials from liquid nitrogen tank and thawing these cryovials in a 37°C water bath for 5 – 10 minutes. The cryovials were very gently flicked and sprayed with 70% ethanol, after which the cryovials were opened in a biosafety hood. These cells were immediately mixed with 10 mL fresh media and transferred to a 15-mL conical centrifuge tubes before they were centrifuged at 1000 rpm for 10 minutes. The supernatant was

aspirated and the cell pellets were resuspended in fresh medium with appropriate supplements. 25 mL fresh medium was generally used for cell cultures in T-75 flasks while 40 mL fresh medium was for cell cultures in T-150 flasks.

Cell enumeration was performed using hemacytometer. Briefly, anchorage-dependent CHO cells were collected in suspension after they were washed twice with PBS and incubated briefly in 0.05% Trypsin/EDTA solution for 5 minutes at 37°C. The cells were stained with trypan blue and diluted appropriately. Non-viable cells were stained blue due to the lack of membrane integrity while viable cells excluded the trypan blue. The cell number per well was determined by counting the stained cells and non-stained cells under microscope and multiplying the concentration by the dilution number. Cell enumeration was done twice per sample and each cell count consisted of at least 200 total cells. Additional cell counts were performed if the duplicate counts did not agree within 10%.

3.2 Sialidase siRNA Method

3.2.1 Optimization of transfection condition

CHO cells were inoculated at a concentration of 1×10^5 cells/well in a 6-well plate 24 hour prior to transfection. The amount of siRNA, the type of RNA carrier, and the amount of RNA carrier were varied depending on the experiment. Nevertheless, the general experimental protocol used was consistent. On the day of transfection, non-silencing fluorescein labeled siRNA (Qiagen, Valencia, CA) was diluted to a final volume of 50 μ L serum-free OptiMEM. Serum occasionally contained RNase that degraded siRNA and thus, the utilization of serum in transfection experiment was avoided. In a separate tube, RNA carrier (either Oligofectamine or

Lipofectamine 2000) was diluted in 50 μ L serum-free OptiMEM. During the incubation period, CHO cell medium was aspirated and the cells were washed twice with PBS after which 900 μ L serum-free OptiMEM was added. 100 μ L of siRNA solution was subsequently added to each well. Each plate was gently shaken immediately after reagent addition. After 4 hours of incubation at 37°C, each well was washed with PBS and fresh DMEM (supplemented with 20% IFS) was introduced. Every 24 hours, a cell count was performed, and cells were collected after incubation with 0.05% Trypsin/EDTA solution for 5 minutes at 37°C. 1×10^6 cells were pooled from numerous wells and diluted in PBS. The cells were centrifuged at 13,000 rpm for 2 minutes and diluted in PBS. This washing procedure was repeated twice before the cell pellet was diluted in 200 μ L PBS. The suspended cells were transferred to 96-well plate and fluorescein fluorescence was measured using a plate reader with appropriate optical filters.

3.2.2 *Sialidase siRNA design*

The Tuschl rules were used to design several siRNA duplexes (Elbashir et al., 2002). In summary, 23-nt sequences with the motifs AA(N₁₉)TT, where N represents any nucleotide with about 50% G/C content, were searched for. The target region was selected 50 to 100 nt downstream of the start codon. In addition, the motifs NN(N₁₉)TT were adopted. The sequence of the siRNA sense strand was designed from (N₁₉)TT sequences while the sequence of the siRNA antisense strand was designed complementary to positions 1 to 21 of the 23-nt motif. All of the siRNAs were ordered from Dharmacon Research, Lafayette, CO. At least 5 sequences from different locations of the cytosolic sialidase cDNA sequence were selected in an experiment. A BLAST search was performed to prevent significant overlap with other CHO

genes from occurring. Fluorescein labeled, non-silencing siRNAs (Qiagen Cat# 1022079) were used as a transfection control.

3.2.3 Preparation of siRNA duplexes

Chemically synthesized 21-nucleotide siRNAs were ordered from Dharmacon Research. The siRNA arrived in the form of water-soluble, stable, 2'-protected single stranded RNA. The 2'-protection ensured that no RNA degradation occurred before use. Two-hundred μL of deprotection buffer (100 mM acetic acid – TEMED pH 3.8) was added to each single-stranded complementary RNA strand. Both strands were mixed by vortexing and were incubated at 60°C for 1 hour. The strand mixture was left for another 1 hour at room temperature to form RNA duplexes. 40 μL of 10 M ammonium acetate and 1.5 mL of 100% ethanol were added to 400 μL of siRNA duplex solution before the final mixture was left at -30 °C for 2 days. The frozen mixture was thawed on ice and centrifuged at 13,000 rpm for 1 hour at 4°C. The supernatant was decanted before the pellet was rinsed with 200 μL of cold 95% ethanol. Then, the pellet was dried under vacuum with speed-vac. The dry pellet was suspended in 1 mL 1x siRNA universal buffer provided by Dharmacon. The siRNA duplex was quantified using an Eppendorf Biophotometer.

3.2.4 Sialidase siRNA transfection and RNA collection

CHO cells were inoculated at a concentration of 1×10^5 /well in a 6-well plate 24 hours prior to transfection. On the day of transfection, 5 nmol of siRNA duplex was diluted to 50 μL in OptiMEM (Invitrogen) and 10 μL of Lipofectamine 2000 was diluted to 50 μL in OptiMEM in

separate tubes. After a five minute incubation at room temperature, diluted siRNA was mixed gently with the Lipofectamine 2000 mixture and incubated for 20 minutes at room temperature. During the incubation period, CHO cell medium was aspirated and the cells were washed twice with PBS after which 900 μ L of serum free OptiMEM was added. One-hundred μ L of siRNA solution was subsequently added to each well. After 4 hours of incubation at 37°C, each well was washed with PBS and fresh DMEM (supplemented with 20% FBS) was introduced. Every 24 hours, a cell count was performed, and cells were collected after incubation with 0.05% Trypsin/EDTA solution for 5 minutes at 37°C. 1×10^6 cells were then washed twice with PBS before they were stored at -20°C for future sialidase assays. RNA was extracted from an additional 1×10^6 isolated cells using a Qiagen RNeasy Mini Kit. RNase free DNase (Qiagen) treatment was performed to remove the residual DNA present in the RNA prep.

3.2.5 Sialidase activity assay

Confluent CHO cells were trypsinized using 0.05% Trypsin/EDTA solution and washed three times with PBS. Then, CHO cells were resuspended in cold water for osmotic lysis and were passed through 26G3/8 needles at least twenty times. 4 mM 2'-(4-methylumbelliferyl) – α – D – N-acetylneuraminic acid (4MU-NeuAc) (Sigma) diluted in potassium phosphate buffer was added to the lysate to a total volume of 100 μ L. At the same time, several dilutions of sialidase (Roche) with known activities were reacted with 4MU-NeuAc as standards. The samples were incubated for 90 minutes at 37°C after which 900 μ L of 0.2M glycine buffer pH 10.4 was added to stop the enzymatic reaction. Two-hundred and fifty μ L of the final solution was transferred to 96-well black plates and fluorescence was measured using a plate reader with an excitation of 362 nm and an emission of 448 nm. Normalization was performed using cellular protein content

determined with a BCA assay kit (Sigma). The sialidase activity assay described in this paragraph was a modification of a method originally developed by Potier and coworkers (Potier et al., 1979).

3.2.6 *Thermodynamic analysis of siRNA sequences*

Asymmetrical thermodynamic analysis was employed based on a work recently done by Schwartz and her colleagues at the University of Massachusetts (Schwartz et. al, 2003). The free energy (ΔG) for the antisense strand of the 21-nt siRNA was estimated by a sum of the energies for 4 base pair stacks and the 3' dangling T for the 5' end of the antisense strand. ΔG for the sense strand was simply the sum for the 5' end of the sense strand. The software implementation of nearest-neighbor estimate and the mfold algorithm was employed to calculate the stability of the initial four base pairs of the siRNA strands (Mathews et al., 1999; Zuker, 2003). The average of internal stability values for positions 9-14 of antisense strand (AIS) was estimated as demonstrated by Khvorova and her co-workers in Amgen and Dharmacon (Khvorova et al., 2003). Sfold software for statistical folding and rational design of nucleic acids was utilized to perform an estimate of the AIS (Ding et al., 2004).

3.3 RNA Analysis

3.3.1 *Total RNA isolation*

All the chemicals required in the total RNA isolation were obtained from the Qiagen RNeasy Mini Kit unless otherwise stated. Briefly, 350 μ L buffer RLT was mixed with β -mercaptoethanol (Sigma) and was introduced to the cell pellet. This mixture was transferred to a Qiashredder spin

column placed in a 2 mL collection tubes and centrifuged at 13,000 rpm for 2 minutes. 350 µL of 70% ethanol was added to the homogenized cell lysate. The ethanol-lysate mixture was loaded into RNaeasy minispin columns provided with the kit and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and the column was subsequently washed once with 350 µL buffer RW1. 10 µL of DNase I stock solution (which consisted of 1500 Kunitz units of DNase I dissolved in 550 µL RNase-free water) was added to 70 µL buffer RDD and this DNase I incubation mix was added to RNaeasy minispin column. After 1 hour incubation at room temperature, the minispin column was washed once with 350 µL buffer RW1 and twice with 500 µL buffer RPE. At the end, the RNA was collected by eluting the column with 50 µL RNase-free water and was stored at -80°C for future uses.

3.3.2 RNA quantification

Total RNA was diluted with RNase free water to reach a dilution of either 10 or 50 fold. The concentration was measured by estimating the absorbance at 260 nm whereby 1.0 OD was estimated to be 40 µg/mL RNA. The quality of RNA was assessed from the ratio of absorbance at 260 nm to the absorbance at 280 nm. A ratio of 1.8 or higher was an indication of high quality RNA. RNA which did not fulfill this criterion was discarded and the isolation protocol was repeated until a ratio of 1.8 or higher was achieved. All the absorbance measurements were performed using an Eppendorf Biophotometer.

3.3.3 First-strand cDNA synthesis

One µg of RNA was thawed on ice and immediately mixed with 1 µL of 50 µM oligo(dT)₂₀ and 10 µM of dNTP mix. DEPC-treated water was then added to a final volume of 10 µL and the whole mixture was incubated at 65°C for 5 minutes followed by brief incubation on ice. Ten µL of cDNA synthesis mix (which consisted of 2µL 10x RT buffer, 4 µL 25 mM MgCl₂, 2 µL 0.1M DTT, 1µL 40U/µL RNaseOUT, and 1 µL 200U/µL SuperScript III RT) was added to the initial mixture. The final solution was incubated at 50°C for 1 hour and 85°C for 7 minutes. 1 µL RNaseH was added. The solution was then incubated at 37°C for 30 minutes.

3.3.4 RT-PCR assay

This first strand reaction product was chilled on ice before the addition of 10 µL 10x PCR buffer without Mg, 1 µL 10 mM dNTP mixture, 1.5 µL 50 mM MgCl₂, 5 µL of 10 µM primer mix, and 0.2 µL 5U/µL Platinum Taq DNA polymerase. Autoclaved water was added to a final volume of 50 µL. The first sense primer for sialidase was 5'-CTTACAGAATCCCTGCTCTGATCTA-3' and the first antisense primer for sialidase was 5'-ATTTGACTCATACAGACACCCAAAT-3'. The second sense primer for sialidase was 5'- GCCTCGGT TAAAAGTGAGAAAAG -3' and the second antisense primer for sialidase was 5'- AGGTAGGCTTGGGTCACCACT-3'. β-actin was used as a control using the sense primer 5'-AGCTGAGAGGGAAATTGTGCG-3' and the antisense primer 5'-GCAACGGAACCGCTCATT-3'. The PCR reaction cocktails were transferred to a 96-well plate and the following thermal cycler program was performed. 1 cycle of (94°C – 2min), 20-35 cycles of (94°C – 30 sec, 55°C – 30 sec, 72°C – 2 min). After the PCR was complete, the product was stored at 4°C and the result was verified by gel electrophoresis.

3.3.5 *Real time RT-PCR (qPCR) assay*

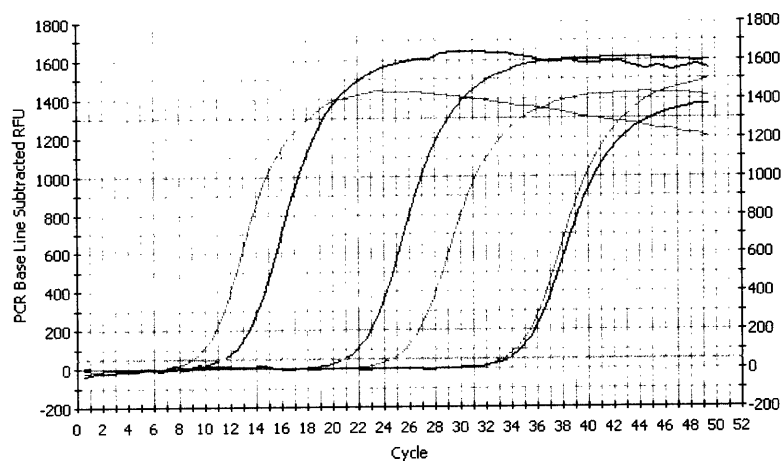
A 100-bp region of the cytosolic sialidase cDNA was amplified using primers VS1F (5'-GGCCTCGGTAAAAAGTGAGAAA-3') and VS1R (5'-GGAGCACGGAGATCATTCCT-3') while a 163-bp region of β -actin cDNA was amplified using primers F3-Actin (5'-AGCTGAGAGGGAAATTGTGCG-3') and R9-Actin (5'-GCAACGGAACCGCTCATT-3'). β -actin was used as internal control with the assumption that β -actin mRNA was constant over most experimental conditions. Primer sequences for sialidase were selected based on trial and error experiments with over 20 pairs of primers. The specificity and efficacy of each primer was assessed by qualitatively observing the gel electrophoresis of the RT-PCR products. Annealing temperatures for both sialidase and β -actin were selected based on a gradient PCR optimization by testing annealing temperature between 50 and 60 °C. Annealing temperature of 55 °C for both genes were selected based on the sharpest and most intense PCR product when run on an agarose gel.

A real time RT-PCR assay was performed in a total reaction volume of 50 μ L. This consists of 25 μ L iQ SYBR green supermix (Bio-Rad, Hercules, CA), 0.4 μ M of each primer, 2 μ L of cDNA obtained from 1 μ g RNA, and RNase free water to reach a total volume of 50 μ L. Real-time PCR was conducted in 96-well plates using the iCycler RT-PCR machine (Bio-Rad). The PCR cycle used a single 3-min hot start at 95°C, followed by 50 cycles of 30 sec at 95°C, 1 min at 60°C, and 2 min at 72°C, during which time the reaction fluorescence was measured. Melting curve analysis was performed by adding a cycle consisting of 1 minute at 95°C followed by

temperature decrease in 1⁰C increments to reach 4⁰C. A 10-second hold was introduced at every temperature decrease.

SYBR Green I dye binds double stranded DNA molecules and produces fluorescent signals on binding. As a result, the fluorescence measured in each amplification cycle is proportional to the amount of double stranded DNA at the end of each amplification cycle. Examples of fluorescence vs. cycle number are shown in Figure 3.1. Sialidase standards and β -actin standards were obtained by amplifying the gene by PCR, cleaning the resulting mixtures, then diluting it to concentrations ranging from 10^{-4} to 10^{-17} $\mu\text{g}/\mu\text{L}$. The R^2 value of the standard curve, relating the threshold cycle to the amount of each standard, was always greater than 0.97. This is shown in Figure 3.2. The specificity of PCR reactions was verified by introducing a melting curve analysis. A single peak without a peak at lower temperatures was an indication of specific amplification reactions. Melting curve analysis of sialidase and β -actin standards at various concentrations is shown in Figure 3.3.

A. IFN γ Standards



B. β -actin standards

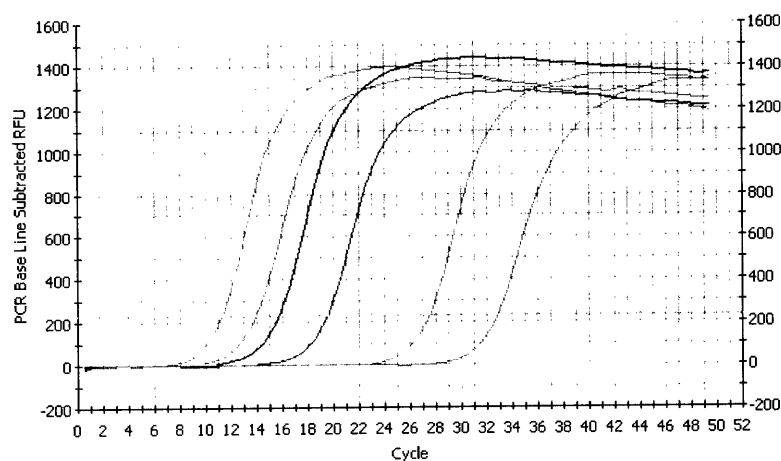


Figure 3.1 Real time RT-PCR fluorescence versus PCR cycle number.

A normalized fluorescence versus PCR cycle number for (A) IFN γ standards and (B) β -actin standards diluted in various concentrations were shown. Threshold fluorescence was automatically calculated by iCycler RT-PCR machine software and was shown as horizontal orange line on the figures.

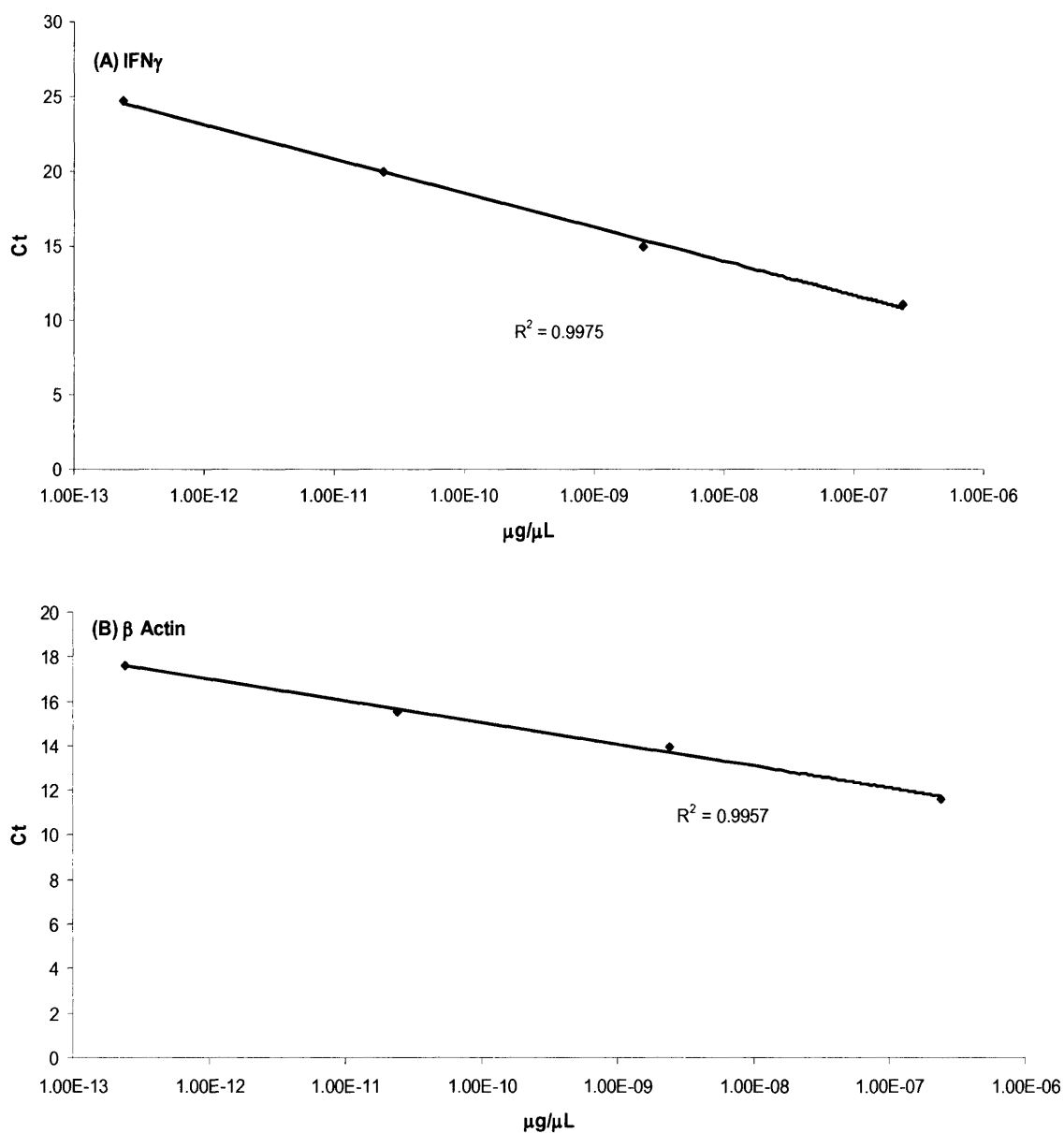


Figure 3.2 Threshold cycle (Ct) as a function of DNA standards concentrations for sialidase and β -actin.

As shown here, the R^2 of standard curve relating the threshold cycle to the amount of dilution of standards for both sialidase and β -actin was always greater than 0.99. Concentrations of each gene in sample were subsequently interpolated from the standard curve.

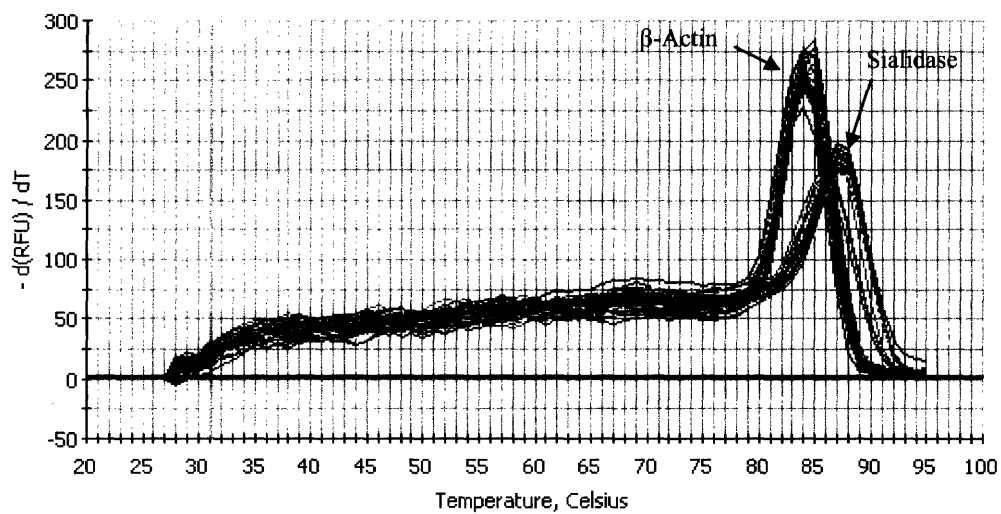


Figure 3.3 Melting curve analysis of sialidase and β -actin standards

By performing melting curve analysis, the amplification of only one product was confirmed. If there were more than one peak in a melting curve, the amplification reaction was not specific. The absence of any small broad peaks at lower temperatures indicated that contaminating products did not exist.

3.4 Stable Cell Line Creation

3.4.1 Plasmid design and transfection

pSilencer encoding a drug selection marker (Hygromycin) was obtained from Ambion (Austin, TX). Three separate promoters (U6, H1, and modified CMV) were utilized in this study. The abbreviated version of the vector maps are shown in Figure 3.4, 3.5, and 3.6. Short hairpin RNA (shRNA) template oligonucleotides were designed based on the sequences that most effectively silenced sialidase, as identified by real time RT-PCR, and fluorescence activity assays. This design was illustrated in Table 3.1. All the oligonucleotides were ordered from Integrated DNA Technologies. 10 ng of annealed shRNA template oligonucleotides were ligated into 0.1 µg pSilencer with 5 units of T4 DNA Ligase (New England Biolabs, Beverly, MA) at room temperature overnight. Ligase was inactivated by heating at 65°C for 10 minutes.

Ligation product was transformed into DH5α competent cells (Invitrogen) according to the instruction manual. Briefly, competent cells were removed from the -80°C freezer and immediately placed on ice. After thawing the cells for about 15 minutes, 1-2 µL of ligated pSilencer plasmid was added to the cells in a 1.5-mL sterile microcentrifuge tube. The tube was subsequently placed on ice for 30 minutes before the cells were heat shocked in 42°C water bath for 45 seconds. Then, the tube was placed on ice for 2 minutes before 900 µL SOC medium was added. The cells suspended in SOC medium were shaken at 37°C incubator for an hour. This solution was diluted 10 times and 100 times before it was spread on the surface of agar plates that were prepared with 50 µg/mL ampicillin. The plates were inverted and incubated at 37°C incubator for 12 – 16 hours. pUC19 plasmid was used as positive control. The next day, a few

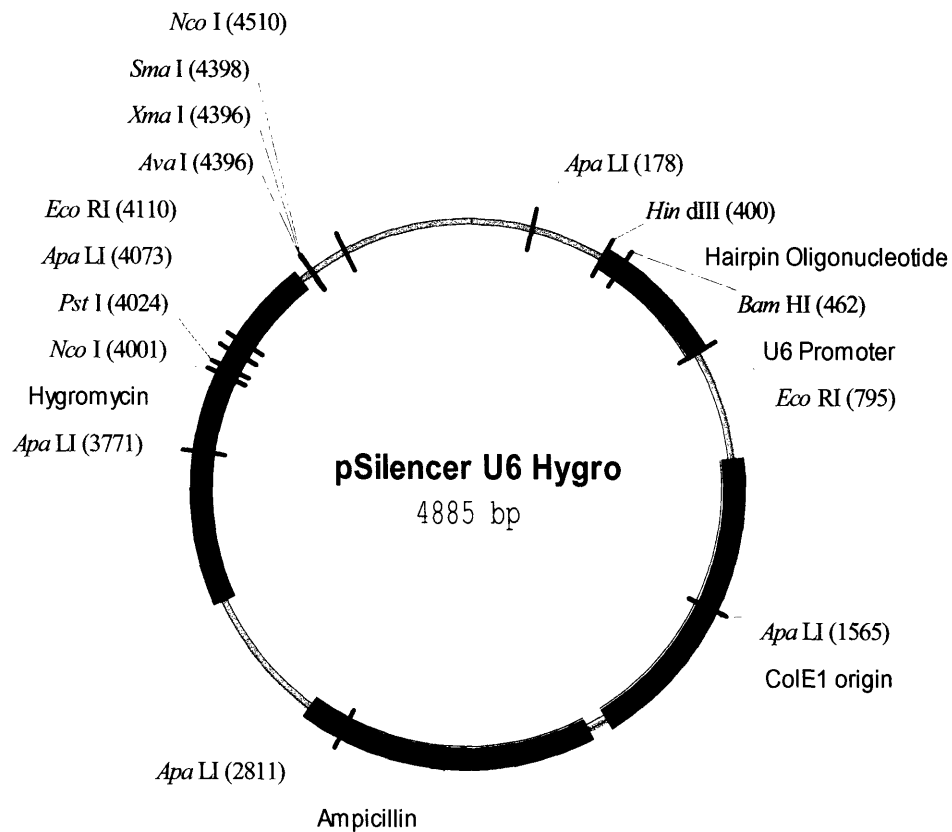


Figure 3.4 Vector map of pSilencer with U6 promoter

The plasmid was linearized with HindIII and BamHI. A hairpin oligonucleotide was subsequently annealed using ligase. Before the plasmid was transfected into CHO cells, linearization at ampicillin site was frequently done.

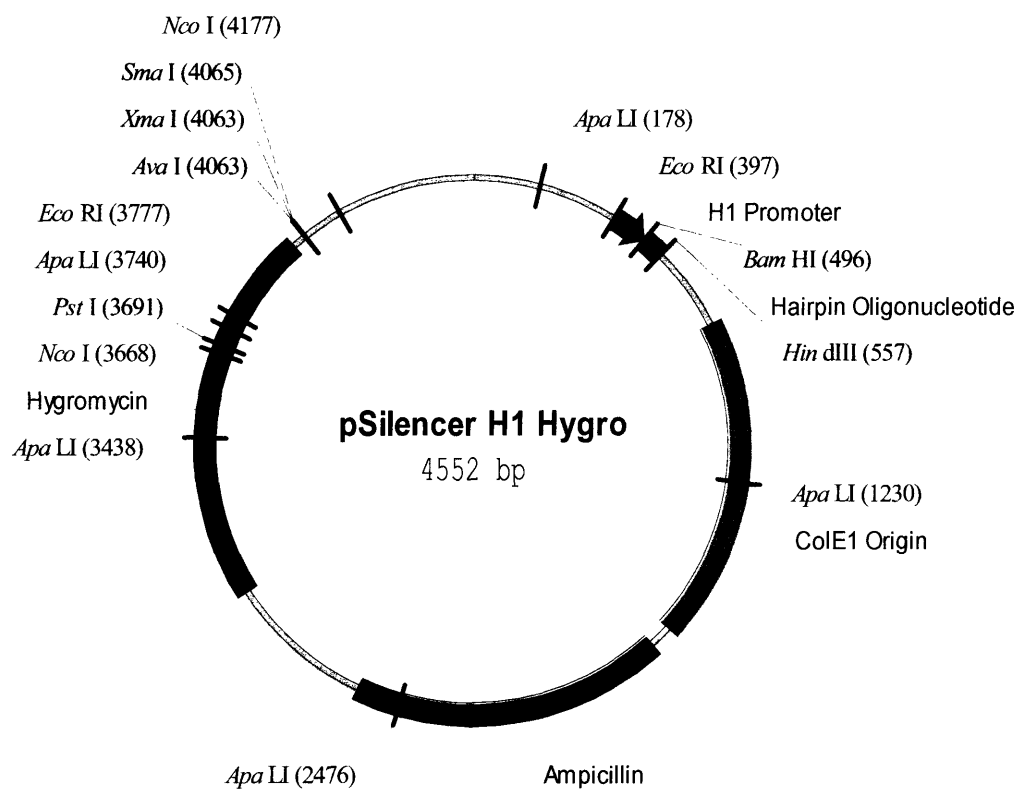


Figure 3.5 Vector map of pSilencer with H1 promoter

The plasmid was linearized with HindIII and BamHI. A hairpin oligonucleotide was subsequently annealed using ligase. Before the plasmid was transfected into CHO cells, linearization at ampicillin site was frequently done.

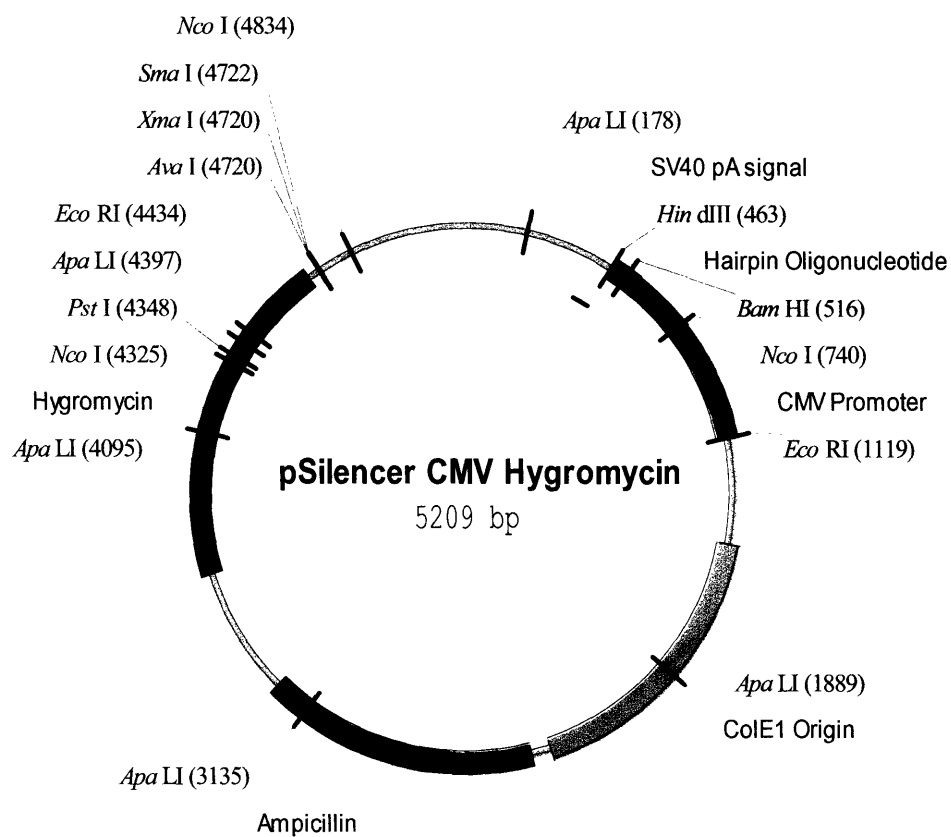


Figure 3.6 Vector map of pSilencer with modified CMV promoter

The plasmid was linearized with HindIII and BamHI. A hairpin oligonucleotide was subsequently annealed using ligase. Before the plasmid was transfected into CHO cells, linearization at ampicillin site was frequently done.

Table 3.1 Sequence of 21-nucleotide siRNA and shRNA template designed to target sialidase mRNA

21-nt siRNAs were designed either using Tom Tuschl's rules (sequence S5) or by selecting randomly across sialidase cDNA sequences (sequence S1). The table above depicted the chemically synthesized 21-nt siRNAs that knocked down sialidase transiently. These sequences were subsequently utilized as templates for short hairpin RNA (shRNA) designed for stable sialidase knock down.

Description	Sequence
Sialidase siRNA Sequence S1	5' A U C A U C U G C A G G G C C U C G G U U 3' 3' U U U A G U A G A C G U C C C G G A G C C 5'
Sialidase siRNA Sequence S5	5' G C C G G U C C U C C C U U C U C C A U U 3' 3' U U C G G C C A G G A G G G A A G A G G U 5'
Sequence S1 CMV Sense (Hairpin Template)	5'-GATCC ATCATCTGCAGGGCCTCGG TTCAAGAGA CCGAGGCCCTGCAGATGATCC A-3'
Sequence S1 CMV Antisense (Hairpin Template)	5'-AGCTT GGATCATCTGCAGGGCCTCGG TCTCTTGAA CCGAGGCCCTGCAGATGAT G-3'
Sequence S5 CMV Sense (Hairpin Template)	5'-GATCC GCCGGTCCTCCCTTCTCCA TTCAAGAGA TGGAGAAGGGAGGACCGGCTT A-3'
Sequence S5 CMV Antisense (Hairpin Template)	5'-AGCTT AAGCCGGTCCTCCCTTCTCCA TCTCTTGAA TGGAGAAGGGAGGACCGGC G-3'

colonies were picked and grown in 200 mL LB medium supplemented with 50 µg/mL ampicillin. A Qiagen HiSpeed Plasmid Maxi Kit was utilized to purify the plasmid. To confirm that the insert was correct and that no mutation occurred, the plasmid was sequenced with the following primer: 5'-AGGCGATTAAGTTGGGTA-3'.

CHO-IFN γ cells were inoculated at density of 1×10^5 /well in a 6-well plate and the shRNA plasmid was linearized with XmnI (New England Biolabs) one day before the transfection. Three µL Fugene 6 (Roche) was added to 97 µL serum-free DMEM. One µg of linearized plasmid was gently mixed with the diluted Fugene and the final mixture was incubated for 30 minutes at room temperature before being added to CHO-IFN γ . Sixteen hours later the medium was replaced with DMEM supplemented with 600 µg/mL Hygromycin. The medium was changed every 3 days and when the confluence reached > 80%, the cells were split. Cloning rings were used to isolate numerous cell colonies, which were then transferred to a 24 well plate. When the confluency reached >80%, the colonies were moved to 6 well plates and then to T-75 flasks.

3.4.2 Screening for Low Sialidase Activity CHO Clones

One million cells from each clone were lysed using the procedures outlined above. Clones with sialidase activity reduced by 50% or greater were passaged a second time and re-assayed. Some clones did not exhibit consistent reduction and were discarded. The remaining clones were passaged and re-assayed. The clones that showed consistent reduction over 3 passages were further characterized.

3.5 IFN- γ Analytical Methods

3.5.1 Purification and quantification of IFN- γ

Supernatant from stable clones and untransfected parent CHO-IFN γ was collected at various time points during cell culture. The supernatant was centrifuged at 1000 rpm for 10 minutes and filtered (0.22 μ m). 50 mL of prepared supernatant was loaded at 0.2mL/min onto an anti-human IFN γ immunoaffinity column that had been equilibrated with loading buffer (20 mM sodium phosphate buffer and 150 mM sodium chloride adjusted to pH 7.2). This purification process was performed on an AKTA Explorer 100 chromatographic system (Amersham Biosciences, Uppsala, Sweden). The column was then washed with loading buffer and the sample was eluted using elution buffer at 0.02 mL/min. The elution buffer consisted of 150 mM sodium chloride adjusted with HCl to pH 2.5. After eluting the samples, the column was regenerated for subsequent runs using loading buffer. Reverse phase HPLC was performed to quantify purified IFN γ . 25-50 μ L purified IFN γ was injected into a Shimadzu LC-10ADvp HPLC (Shimadzu Analytical instruments, Kyoto, Japan) and separated on a Vydac C18 1mm x 250 mm column (Grace Vydac, Hesperia, CA). The sample was eluted over a 30 min linear gradient from 35% (v/v) to 65% (v/v) buffer B (buffer A: HPLC grade water + 0.1% trifluoroacetic acid (TFA), buffer B: HPLC grade acetonitrile + 0.1% TFA) at a flow rate of 0.05 ml/min. Eluted IFN γ was monitored at 220 nm and quantified by comparing the samples with standards of known IFN- γ concentration.

3.5.2 Sialic acid content analysis of IFN- γ

A modified version of the thiobarbituric acid assay (TAA) was used to measure the amount of total sialic acid per protein (Hammond and Papermaster, 1976). Sialic acid from 3-6 μ g of

purified IFN- γ was cleaved by incubating IFN- γ with 0.0025U sialidase (Roche) for 24 hours at 37°C. After digestion, water was added to yield a final volume of 500 μ L. Two-hundred and fifty μ L of periodic acid reagent (25 mM periodic acid in 0.125N H₂SO₄) was added to this mixture, which was then incubated at 37°C for 30 minutes. Excess periodate was destroyed by adding arsenite solution (200 μ L of 2% sodium arsenite in 0.5N HCl) before 2 mL of thiobarbituric acid reagent (2 mL of 0.1M 2-thiobarbituric acid, adjusted to pH 9 with NaOH) was added. The solution was heated at 98°C for 7.5 min then incubated on ice for 10 minutes. One and a half mL of acid/butanol solution (n-butanol with 5% (v/v) 12N HCl) was added to the cooled sample and the mixture was shaken vigorously before it was centrifuged at 3,000 rpm for 3 minutes. The clear organic phase was transferred to a 10 mm cuvette and the fluorescence intensity ($\lambda_{\text{ex}} = 550$ nm, $\lambda_{\text{em}} = 570$ nm) was measured with a Cary Eclipse Fluorescence Spectrophotometer (Varian Inc, Palo Alto, CA). A standard curve generated from pure sialic acid samples was used to quantify the sialic acid content from each sample. The assay was repeated for three times.

3.5.3 Glycan site occupancy analysis of IFN- γ

Site occupancy analysis of three IFN- γ glycoforms was performed using Micellar Electrokinetic Capillary Chromatography (MEKC) on a Beckman Coulter P/ACETM MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA). A 50 μ m diameter x 52 cm (40 cm length to detector) unfused silica capillary (Beckman Coulter) was used for the separation. The capillary was initially cleaned with 0.1M NaOH for 15 minutes, flushed with HPLC grade water for 10 minutes, and subsequently equilibrated with running buffer for 15 minutes. The running buffer consisted of 30 mM sodium borate, 30 mM boric acid and 100 mM SDS at pH 9. Samples

were pressure injected at 3 psi over 10 second and then 12-15 kV voltage was applied to the capillary over 60-80 minutes. The chromatograms were integrated to quantify the percentage of 2-site, 1-site, and nonglycosylated peaks.

3.5.4 *Microheterogeneity analysis of IFN γ*

3.5.4.1 Trypsin and PNGase F digestion

Purified IFN- γ (50 – 100 μ g) was diluted with digestion buffer (50 mM ammonium bicarbonate adjusted to pH 8.5) to a final concentration of 0.1 μ g/ μ L. Lyophilized sequencing grade modified trypsin (Promega) was dissolved in digestion buffer to a final concentration of 0.04 μ g/ μ L. The trypsin solution was mixed with the diluted IFN- γ in a 1:20 trypsin-to-protein mass ratio. This mixture was incubated in a water bath at 37°C for overnight. Heat inactivation of trypsin was performed by incubating the mixture at 95°C for 30 minutes. Thirty five units of PNGase F (Calbiochem, San Diego, CA) were subsequently added for every 100 μ g of IFN γ before incubation at 30°C for overnight was performed. Glycan mixtures from PNGase F digestion step was further cleaned up using GlycoClean H cartridges (Prozyme, San Leandro, CA) according to the manufacturer's instructions.

3.5.4.2 Glycan analysis using high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Glycans obtained from trypsin digestion and PNGase F treatments of purified glycoproteins were separated using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Hermentin et.al., 1992). Chromatography separation was performed with CarboPac columns (Dionex Corporation, Sunnyvale, USA) on the Dionex BioLC system

(Dionex) according to the manufacturer's protocol. Pre-optimized glycan standards (Dextra Laboratories, Reading, UK) were run to identify resolved glycan peaks of each sample by matching the retention times. In addition, raffinose standard was used as internal reference for all runs. The spectra of glycan analysis using HPAEC-PAD method was shown in Figure 3.7.

3.5.4.3 Glycan analysis using MALDI/TOF mass spectrometry

Prior to mass spectrometry analysis step, permethylation of the glycans was performed to enhance signal strength (Zaia, 2004). The protocol of glycan permethylation was adapted from Dell et al. (1993). Lyophilization of glycan samples was initially performed using Freezemobile 5EL (VirTis, Gardiner, NY) before sodium hydroxide/dimethylsulfoxide slurry was added to each sample. Half a milliliter of methyl iodide was subsequently mixed to the sample. After continuous agitation for 10 minutes at room temperature, one milliliter of water was added in dropwise to quench the reaction. The solution was constantly shaken before one milliliter of chloroform was added. Water was added to a final volume of 5 mL and the final mixture was allowed to phase separate. The upper aqueous layer was disposed while the chloroform layer was washed repeatedly with water and dried under nitrogen stream. The permethylated mixture was purified with a Sep-Pak C18 column (Waters, Milford, MA) and lyophilized.

Thirty μL of 80% (v/v) methanol was used to redissolve lyophilized permethylated glycans. Equal volumes of permethylated samples and 2,5-dihydroxybenzoic acid (10 mg/mL 2,5-dihydroxybenzoic acid dissolved in 80% (v/v) methanol) was loaded onto MALDI plate and analyzed with Voyager DE-STR Biospectrometry system (Applied Biosystems). Acceleration voltage of 20 kV was used to accelerate ions after 200 nsec delay time. Data for 100 pulses of the

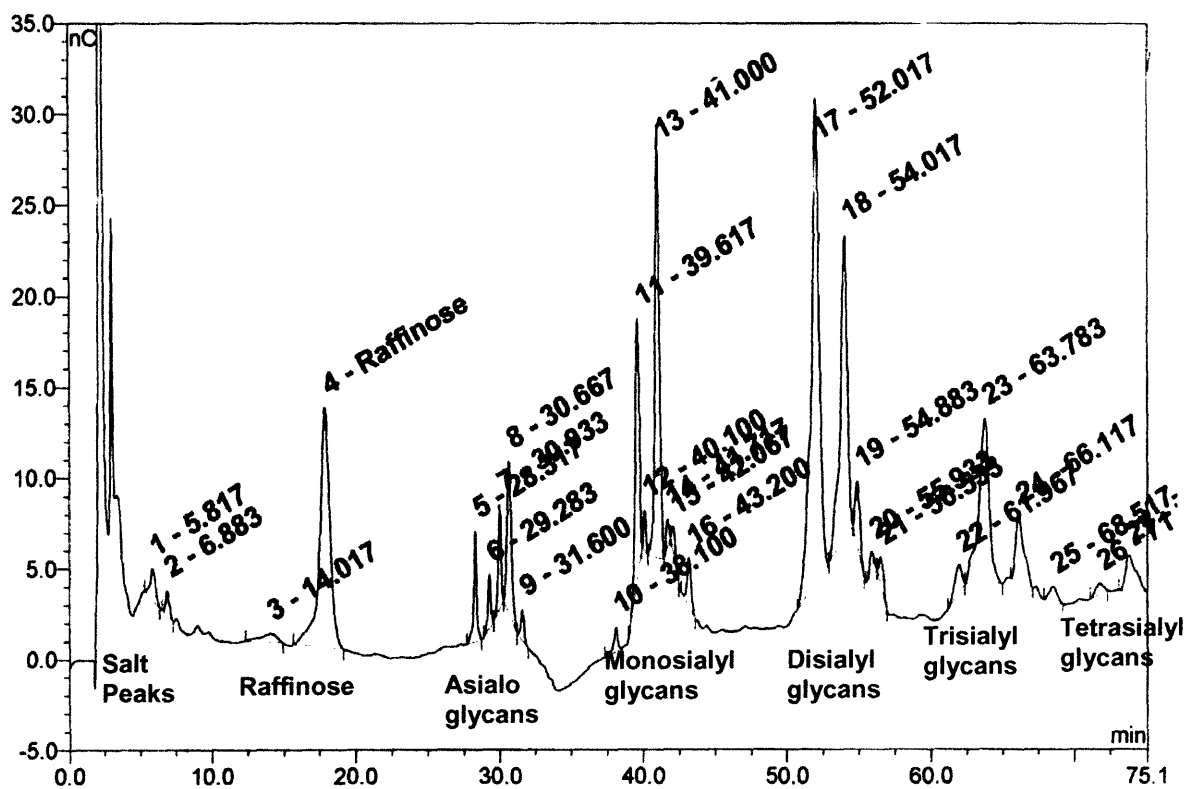


Figure 3.7 High performance anion exchange chromatography spectra of interferon-gamma glycans. In every run, raffinose was used as internal standard.

377 nm nitrogen laser was averaged for each spectrum and detected by a reflectron, positive-ion TOF mode. Voyager v5.1 software (Applied Biosystems) was used to analyze the data.

3.6 Other Analytical Methods

3.6.1 Determination of IFN- γ concentration

IFN- γ concentrations were measured using a commercially available ELISA kit (Biosource International Inc.). On the day of the measurement, the frozen supernatant samples were thawed from -80°C freezer and were diluted with PBS to ensure the samples were within the range of the standard curve (325 – 5000 pg/mL). Every sample was measured in triplicate.

3.6.2 Glucose, glutamine, ammonia, and lactate Concentration Measurement

Supernatant samples from cell culture were centrifuged at 13,000 rpm for 2 minutes to remove cell lysate. The samples were diluted with Milli-Q Ultrapure water before each measurement to ensure the measurement lied within the linear range of the calibration curve for glucose, glutamine, ammonia, and lactate. YSI Biochemistry Analyzers unit was used to measure the diluted samples.

3.7 Construction of GFP-based shRNA Plasmid

3.7.1 *GFP-based screen for cells expressing high copy numbers of pol III-driven sialidase siRNA plasmid construct*

Cloning entry vector pEN_mH1c (ATCC ID: 10326369) and gateway destination vector pDS_hpCG (ATCC ID: 10326383) were generous gifts from Dr. Iain Fraser (Caltech). These vectors were propagated in ccdB resistant *E.coli* cells (Invitrogen Cat#: C7510-03). The design for the shRNA oligonucleotide template was based on sialidase siRNA sequence S1. Sense oligonucleotide sequence is: 5'-GATCCCC ATCATCTGCAGGGCCTCGG TTCAAGAGA CCGAGGCCCTGCAGATGAT TTTTTC-3'. Antisense oligonucleotide sequence is: 5'-TCGAGAAAAA ATCATCTGCAGGGCCTCGG TCTCTTGAA CCGAGGCCCTGCAGATGAT GGG-3'. Sialidase siRNA sequence S1 was shown in red font.

10 µg entry vector pEN_mH1c was incubated with 50 U BamHI and 50 U XhoI for 1.5 hours. After heat inactivation at 65°C for 20 minutes and gel purification, 0.1 µg of linearized pEN_mH1c was combined with 8 ng annealed shRNA template, 5 U T4 DNA ligase, and 1 µL T4 DNA ligase buffer. Nuclease free water was added to reach a final volume of 10 µL. Ligation was performed overnight before the final ligation product was transformed into DH5α competent cells (Invitrogen) as described in the instruction manual. The final product was called pEN_mH1-shRNA.

Site specific LR recombination was performed by initially mixing 150 ng of entry vector pEN_mH1-shRNA with 150 ng of gateway destination vector pDS_hpCG in TE buffer pH 8.0. 4

μL of LR Clonase II enzyme (Invitrogen, Cat#: 11791-020) was thawed on ice for 5 minutes before being added to the vector mix. The final mixture was vortexed and incubated at room temperature for 2 hours. 1 μL of Proteinase K was added to stop the reaction and the samples were incubated at 37°C for 20 minutes before the LR recombination product was transformed into DH5α competent cells. The construction of this GFP-shRNA plasmid is summarized in Figure 3.8. Transfection of GFP-shRNA plasmid with neomycin selection was performed using lipid-mediated transfection method. After transfected cells were subjected to neomycin for 10 – 16 weeks, about 10 – 30 million cells went through fluorescence activated cell sorting (FACS). Subclones were collected based on the top 10% mean GFP fluorescence, the next 20%, another 20%, and the bottom 50% mean GFP fluorescence. A sialidase assay was performed utilizing 4MU substrate and sialidase mRNA was quantified using real-time PCR as explained in previous sections

3.7.2 GFP-based screen for cells expressing high copy numbers of pol II-driven sialidase siRNA plasmid construct

10 μg of pSilencer 4.1-CMV (Ambion) plasmid that has a hairpin template for sialidase sequence S1 cloned in it was linearized by incubating the plasmid with 50 U NarI and 50 U NotI for 1.5 hours. After heat inactivation at 65°C for 20 minutes, the linearized plasmid was gel purified and dephosphorylated with antarctic phosphatase (New England Biolabs). A variant of green fluorescent protein (GFP) was amplified from plasmid pEGFP-C1 (BD Biosciences Cat# 6084-1) using the 5' primer 5'-GCGGCCGCTAGTTATTAATAGTAATCAATTACGGG-3' and the 3' primer 5'-GGCGCC AGGGTTATTGTCTCATGAGCGGAT-3'. These primers were chosen to introduce a NarI site and NotI site at the end of the PCR product. PCR utilizing Platinum Pfx

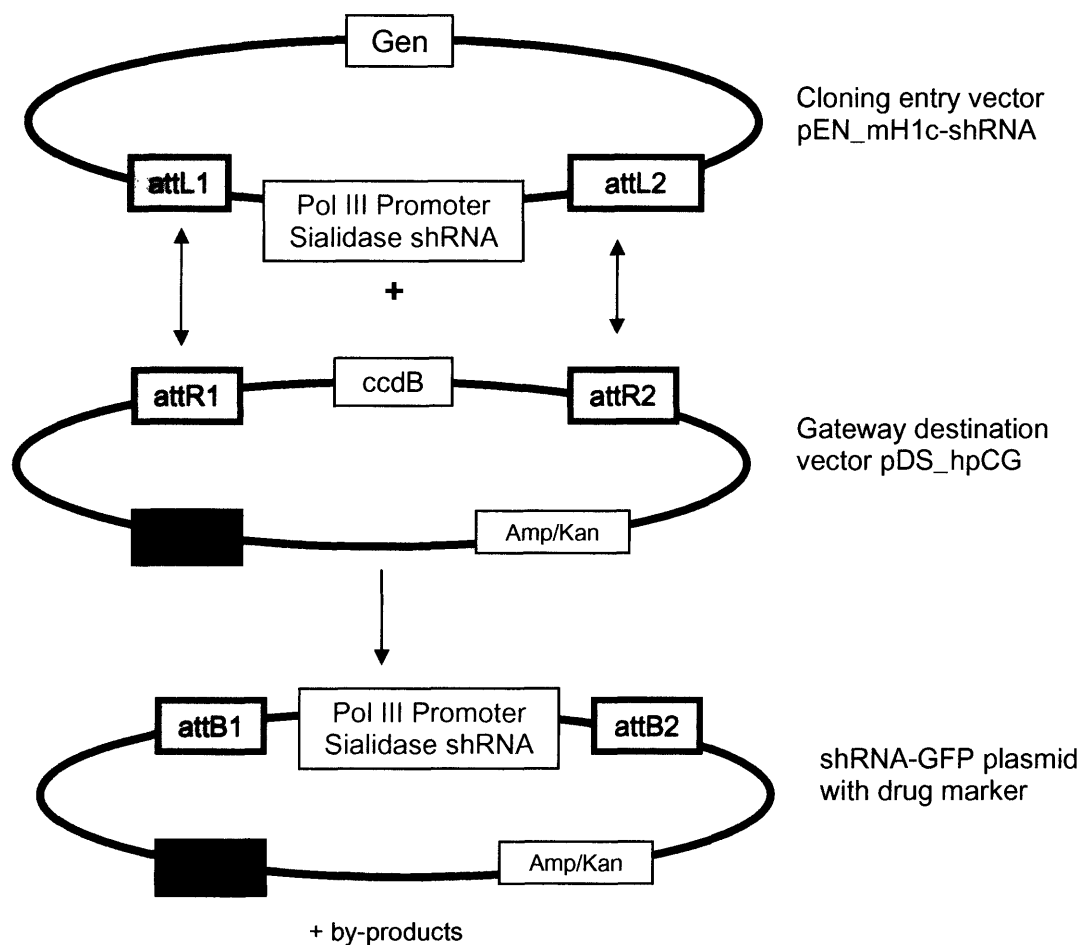


Figure 3.8 Schematic diagram of site specific recombination process to engineer Pol III-driven shRNA-GFP plasmid bearing drug resistance marker

Sialidase sequence S1 shRNA template was initially cloned into pEN_mH1c plasmid. Then, LR recombination reaction between pEN_mH1c-shRNA plasmid and GFP-containing pDS_hpCG plasmid was performed utilizing Gateway technology technique (Invitrogen Cat# 11791-020). Recombination product was transformed into DH5- α competent cells.

polymerase was performed with the following thermal cycler program: 1 cycle of (94°C – 5min), 30-40 cycles of (94°C – 15 sec, 55°C – 30 sec, 72°C – 2.5 min). Gel purified-PCR product was digested with NarI and NotI before it was cloned into the NarI and NotI site of linearized sialidase sequence S1-containing pSilencer 4.1-CMV plasmid (Figure 3.9). Transfection of GFP-shRNA plasmid with hygromycin selection was performed as detailed in previous section. After transfected cells were subjected to hygromycin for 10 – 16 weeks, about 10 – 30 million cells went through fluorescence activated cell sorting (FACS). Subclones were collected based on the mean GFP fluorescence. A sialidase assay was performed utilizing 4MU substrate and sialidase mRNA was quantified using real-time PCR as explained previously.

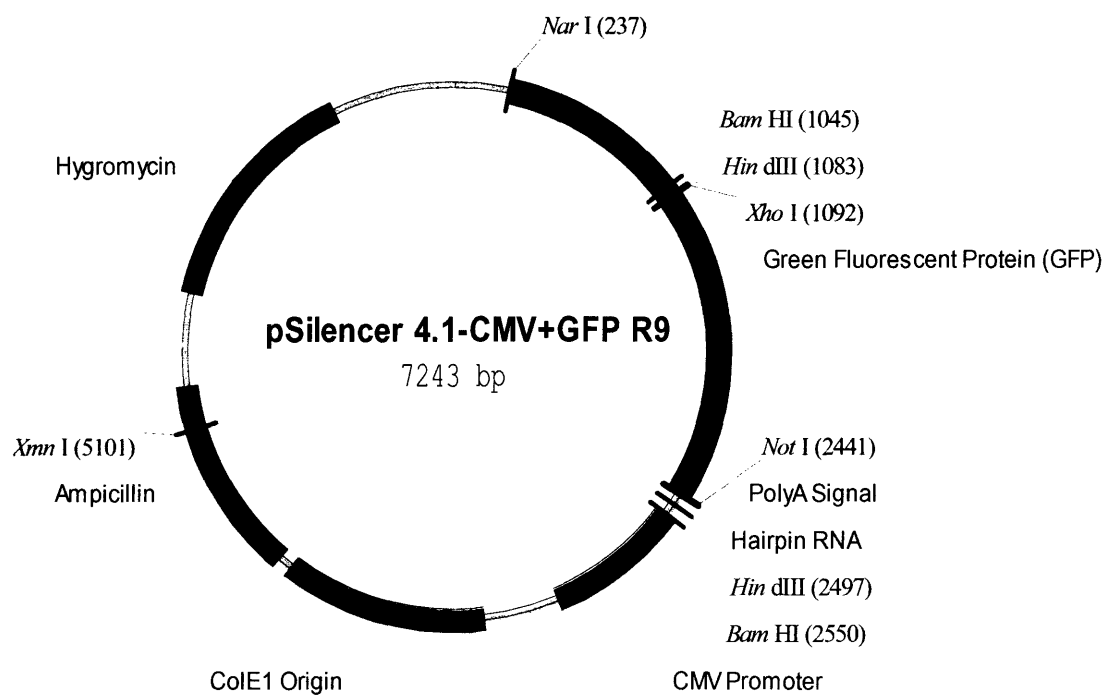


Figure 3.9 Schematic diagram of pol II-driven pSilencer 4.1-CMV plasmid that contains sialidase hairpin RNA template and GFP sequence.

Sialidase sequence S1 hairpin template was initially cloned into pSilencer 4.1-CMV plasmid. Then, GFP fragments amplified from pEGFP-C1 was introduced into linearized pSilencer 4.1-CMV-shRNA at *Nar*I/*Not*I site.

4. Initial Investigation on Exploiting RNA Interference Technique to Reduce Sialidase Degradative Activity

4.1 Introduction

Soluble cytosolic sialidase secreted by dead cells has been shown by many researchers to be one of the causes for the extracellular desialylation of glycoproteins produced by mammalian cells (Gramer and Goochee, 1993; Gramer et al., 1995; Munzert et al., 1996). Before a sialidase gene knock down to deactivate degradative activity study was performed, an *in vitro* enzyme kinetics experiment was conducted with three major reasons. First, it was important to observe if sialidase even existed within the CHO cell lines that would further form the basis for this thesis. In particular, CHO-IFN- γ would have to be characterized first since this cell line was demonstrated to lose sialic acid content as cells lost their viability during prolonged culture time (Gu et al., 1997). 2'-(4-methylumbelliferyl) – α – D – N-acetylneuraminic acid (4MU-NeuAc) artificial substrate was used to quantitate sialidase activity from cell lysate because sialic acid removal could easily be detected by measuring the fluorescence of cleaved substrate. Second, it is necessary in attempting to inhibit soluble cytosolic sialidase activity with 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (2,3 D), a commonly used chemical inhibitor. By performing this experiment, the possibility of competitively inhibiting sialidase was to be confirmed before conducting permanent inhibition via genetic engineering. Finally, by measuring the amount of sialic acid released from fluorimetric and glycoprotein substrates by sialidase, the severity of the desialylation process could be estimated.

When employing RNAi technique to silence sialidase activity, there are two key issues that must be resolved. First, it is crucial to ensure 21 nucleotide sialidase siRNAs are transported into the

cells. Otherwise, it would be hard to distinguish failure in the efficacious gene-specific sequence selection and unobservable knock down due to low transfection efficiency. There are a number of key variables that have the potential to affect the efficiency of gene-specific siRNA sequence delivery. They are cell density, method of transfection, amount of gene-specific 21 nucleotide siRNAs transfected, and exposure time. When doing this optimization study, fluorescein-labeled non-silencing siRNA was initially utilized as a double stranded RNA molecule to be delivered into Chinese Hamster Ovary (CHO) cells because optimal transfection condition can be easily determined by using fluorescence plate reader. Once the optimal condition is found, a negative control can be incorporated for any transfection experiments to assess the potency of different 21 nucleotide siRNA designs.

Another issue of importance would be the selection of the sialidase 21 nucleotide siRNA sequence. Cytosolic sialidase cDNA consists of 1366 bp nucleotides and stochastically there are 1345 possible sequences that could potentially interfere sialidase mRNA. As demonstrated by many researchers, targeting different sites of a gene yielded different gene-specific silencing effects (Elbashir et al., 2001b; Khorova et al., 2003; Miyagishi and Taira, 2002; Reynolds et al., 2004; Ui-Tei, 2004). At the beginning of this project, the only commonly used method to pick siRNA sequences was Tom Tuschl's empirical rule (Elbashir et al., 2001c, 2002). The empirical design rule can be summarized as follows:

1. 21-nt sense and 21-nt antisense paired in a manner to have 2-nt 3' overhang are the basic design.
2. Target region should be chosen 50 – 100 nt downstream of start codon. 5' or 3' untranslated (UTR) regions and regions close to start codon should be avoided as UTR-binding proteins

and/or translation initiation complexes might interfere with binding of RISC to the target RNA.

3. Based on the cDNA sequence of the gene of interest, a 23-nt sequence motif AA(N₁₉)TT where N is any nucleotide is searched. 50% G/C content is ideal while 30% - 70% G/C ratio often works.
4. If no suitable sequence is found, the search is extended using the motif NA(N₂₁). Then, the 3' end of the sense is converted to TT (Elbashir, 2001b).
5. Antisense siRNA is designed as the complement to position 1 to 21 of the 23 nt- motif. The 3' most nucleotide residue of the antisense siRNA can be deliberately chosen but TT is preferred.
6. The potential design must be compared to the appropriate genome database of the close proximate of the animal cells to ensure that only one gene is targeted. This is done by performing BLAST search. 21-nucleotide RNAs can then be ordered from commercial RNA oligo synthesis suppliers.

Following Tuschl's rule does not necessarily guarantee success in finding efficacious siRNA. In fact, many of the effective siRNAs were found randomly. Randomly selected siRNAs yielded knockdown $\geq 50\%$ with 58-78% success rate while very effective siRNAs (defined as those with $\geq 90\%$ knockdown) were found by chance 11-18% of the time (Khvorova et al., 2003; Reynolds et al., 2004). In this analysis, a few siRNA sequences were picked following Tuschl's rule and a few others were picked randomly across various different locations within the sialidase cDNA sequence. Success in knocking down sialidase mRNA would correlate to a lower quantity of sialidase mRNA as analyzed by RT-PCR and/or quantitative PCR. This, in turn, should lead to a

decrease in sialidase activity, which could be measured using methylumbelliferyl fluorescence substrate.

Interfering RNA using chemically synthesized 21-nt siRNA can only provide transient silencing to the transfected mammalian cells. This was because only a limited amount of siRNA was supplied to the cells. Once exhausted, more RNA must be transfected to provide continuous silencing. In addition, as long as there are sufficient nutrients within the growth environment and there is space to grow (i.e. there is room to attach for anchorage-dependent cells), cells will keep proliferating. There will also be a dilution effect that will correlate to the inability to continuously interfere sialidase mRNA in the progeny. A stable silencing system will be needed once an effective sialidase siRNA is found.

4.2 Results and Discussions

***In vitro* Kinetics Analysis of Sialidase Desialylation Process**

Sialidase originating from CHO cell lysate was capable of cleaving sialic acid and exhibited saturation kinetics like many other enzymes (Figure 4.1). Competitive inhibition of sialidase activity using 2,3D was demonstrated to be viable strategy to reduce sialidase activity by 42-89 folds (Figure 4.1). Kinetic parameters estimated from fitting Michaelis-Menten saturation kinetics equation to three independent experiments were comparable to kinetics parameters obtained by other researchers. From Lineweaver-Burk plot construction based on data in Figure 4.1, K_m for sialidase desialylation utilizing 4MU-NeuAc substrate was estimated to be 0.47 mM while other researchers found K_m value using 4MU-NeuAc substrate was in the range of 0.03 – 0.37 mM (Conzelmann and Sandhoff, 1987, Gramer and Goochee, 1993). There were no suitable

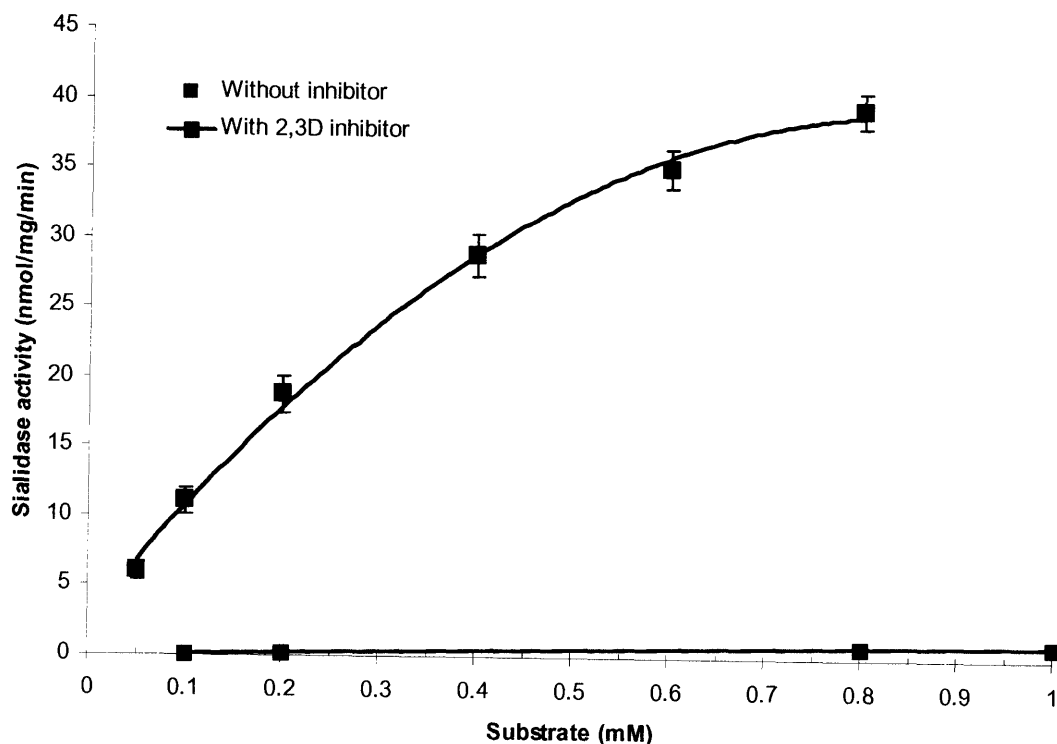


Figure 4.1 Effect of substrate concentration on the rate of sialidase desialylation reaction

Sialidase activity of cell lysate (lysed from 1×10^7 CHO-IFN- γ cells and normalized per mg of cellular protein) was measured using 4Mu-NeuAc fluorescence artificial substrate at different substrate concentrations. Normalization was performed using cellular protein content determined with a BCA kit. A similar experiment was repeated with the introduction of 2,3D inhibitor to confirm the possibility to competitively inhibit sialidase activity. The plot shows the average from three experiments with ± 1 standard deviation. Standard deviations from inhibition study were not apparent in the plot because of their small magnitude.

comparisons for V_m values for desialylation utilizing 4Mu-NeuAc substrates because other researchers computed V_m values on different substrates. Experiments utilizing Cowper's gland mucin estimated V_m to be 0.112 $\mu\text{mol}/\text{min}/\text{mg}$ while experiments utilizing mixed brain gangliosides estimated V_m to be 5.1 $\text{nmol}/\text{min}/\text{mg}$ (Srivastava and Abou-Issa, 1977, Conzelmann and Sandhoff, 1987). These range of values, although not on the appropriate substrates, provided an estimate of the sensible V_m . In our experiment, V_m was estimated to be 62.4 $\text{nmol}/\text{hr}/\text{mg}$, which was within the bound of the estimated V_m by other researchers.

In our future studies of silencing sialidase degradative activity, IFN γ will be used as model recombinant protein. However, no published results were available on the kinetics of desialylation of interferons by cytosolic sialidase. Fortunately, kinetic parameters of IFN γ desialylation could easily be quantitated by performing *in vitro* experiment utilizing cell lysate as the source of sialidase. Instead of quantifying the release of fluorescence substrate, sialic acid cleaved from glycoprotein was monitored using thiobarbituric assay as described by previous researchers (Gramer and Goochee, 1993). From Lineweaver-Burk plot construction shown in Figure 4.2, V_m for desialylation utilizing IFN γ substrate was estimated to be 38.05 $\text{nmol}/\text{hr}/\text{mg}$, which is 40% lower than V_m for desialylation utilizing 4MU-NeuAc substrate. This reduced V_m value might be explained by the different linkage of sialic acid in each substrate. When sialidase cleaved sialic acid attached as 4MU-NeuAc substrate, the only linkage to be broken was a simple, steric-hindrance free link between 4MU and NeuAc. On the other hand, sialic acids on glycoproteins were attached as α -2,3-linked sugars on the sugar chain. The existence of other larger-size carbohydrates and proteins could contribute steric hindrance that will reduce the rate

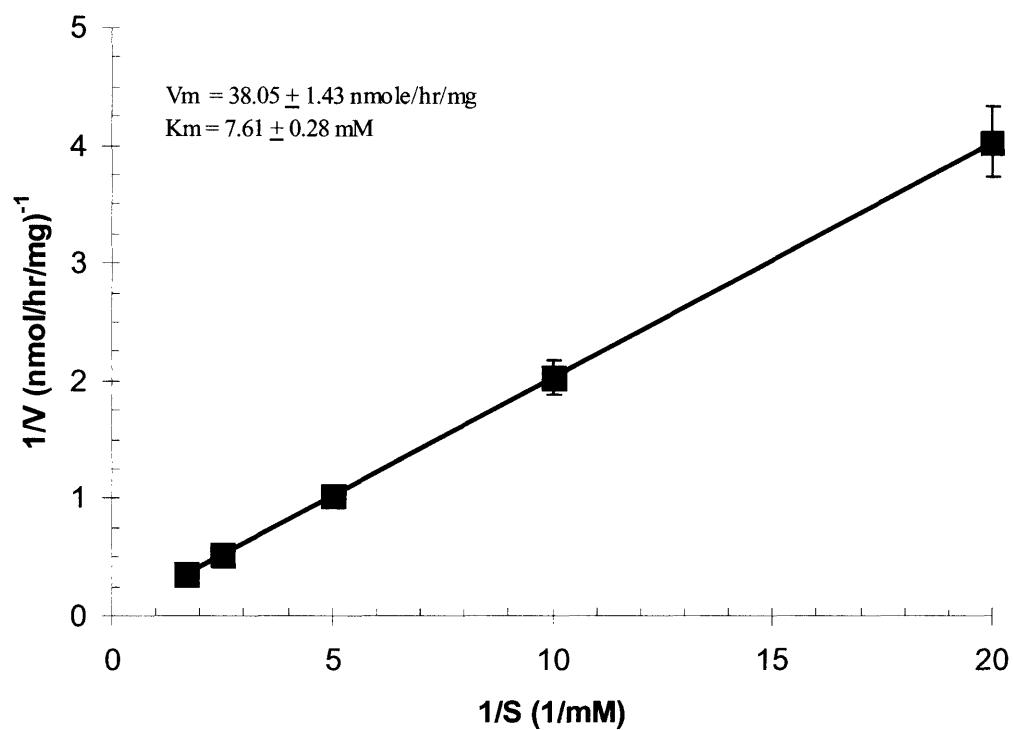


Figure 4.2 Lineweaver-Burk plot of in vitro sialidase desialylation reaction at various IFN γ glycoprotein concentrations

A double-reciprocal plot was constructed to estimate kinetic parameters of sialidase desialylation reaction. Cell lysate was utilized as a sialidase source to cleave IFN γ . All the experiments were performed in triplicate. V_m and K_m displayed on the figure were estimates from three different experiments.

of desialylation. K_m for desialylation utilizing IFN γ substrate was estimated to be 7.61 mM, which was an order of magnitude greater than the K_m utilizing 4MU-NeuAc substrate. This difference represented the various affinities of the enzymes for each substrate.

Based on the estimated *in vitro* kinetic parameters (Table 4.1), whether sialic acid degradation by sialidase was a major issue or not could be assessed. In the culture of CHO-IFN- γ , the typical titer of a batch culture was 3 $\mu\text{g/mL}$ and every mole of biantennary IFN γ could contain a maximal of 4 moles of sialic acid (Yuk, 2001). Assuming Michaelis-Menten kinetics was applicable, the release of sialidase to supernatant as the cells died was estimated to cleave sialic acid at the maximum rate 1.53 nmole of sialic acid/day. This imposed a time limit on how long a batch or fed-batch culture could be run before the quality of glycoprotein deteriorated.

The previous calculation may not be totally accurate because Michaelis-Menten kinetics assumed a constant amount of enzyme. As the cells died, more sialidase can be released into the supernatant. At the same time, as the sialylated glycoprotein was degraded by sialidase, there would be less substrate for degradation. Competitive inhibition by other glycoproteins in the supernatant would not be expected to slow sialidase from cleaving sialic acid off of IFN γ because IFN γ concentration was much higher than other glycoprotein concentrations as a result of methotrexate amplification. In a serum-supplemented culture, fetal bovine serum contained many glycoproteins such as fetuin, albumin, and transferrin (Jayme and Smith, 2000). Although these glycoproteins were shown to be desialylated from *in vitro* experiments by sialidase, they would not serve as competitive inhibitor because of their low abundance as compared to glycoproteins amplified by methotrexate. In addition, the K_m values for these glycoproteins were

Table 4.1 Sialidase kinetic constants estimated from reacting CHO-IFN- γ cell lysate with various sialylated substrates

A double-reciprocal plot was constructed to estimate kinetic parameters of sialidase desialylation reaction. Cell lysate was utilized as a sialidase source to cleave 4-MU NeuAc substrate and IFN γ . All the experiments were performed in triplicate. V_m and K_m displayed on the table were estimates from three different experiments.

Substrate	V_m (nmol/hr/mg)	K_m (mM)
4-MU-NeuAc	62.40 ± 1.58	0.47 ± 0.06
IFN γ	38.05 ± 1.43	7.61 ± 0.28

reported to be on the same order of magnitude with our measured K_m values for IFN γ (Gramer et al., 1995). As a result, competitive inhibition by other glycoproteins in the serum would not be expected to significantly impede desialylation of glycoprotein amplified by methotrexate.

From this study, we have confirmed the existence of sialidase activity originating from CHO cell lysate. Since the experiments were conducted at a pH of 7, which was the typical cell culture environment, it was expected that sialidase, once released into the cell supernatant, could cleave sialic acid off of glycoproteins. Competitive inhibition was found possible using the commercially available inhibitor and applying this principle genetically should be a viable option.

Transfection Optimization

Every cell line exhibited different optimal conditions for transfection of siRNA. Therefore, various factors were explored in this study using fluorescein-labeled siRNA before proceeding with the study of various sialidase siRNAs. Increasing the dose of siRNA was found to be effective in increasing the amount of siRNA delivered into the cells regardless of the type of lipid carrier used (Figure 4.3). This was certainly true holding the amount of lipid carrier constant. It was found that Lipofectamine 2000 was a better transfection reagent than Oligofectamine although Lipofectamine 2000 compensated cell viability. In figure 4.3, saturation of siRNA delivery was attained as the fluorescein-labeled siRNA was doubled from 200 pmole to 400 pmole.

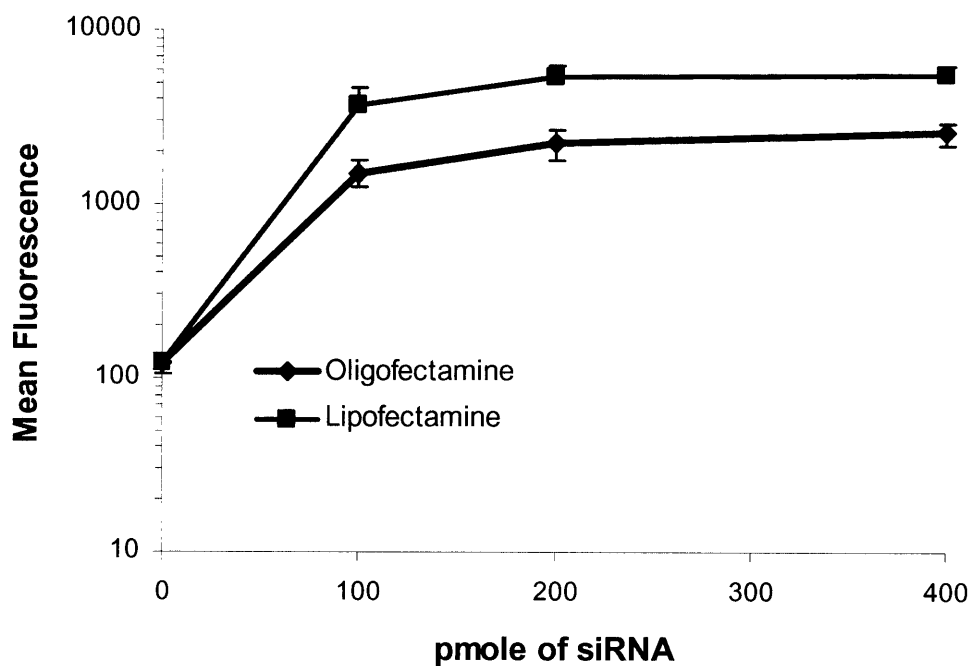


Figure 4.3 Evaluation of various transfection reagents effectiveness in delivering fluorescein-labeled non-silencing double stranded RNA

Different doses of siRNA were transfected into CHO-IFN γ using two different transfection reagents: Oligofectamine (Invitrogen) and Lipofectamine 2000 (Invitrogen). The quantity of transfection reagent was set to be constant at 1 μ L. Error bars illustrate the standard deviations obtain from four cultures.

There were two possible reasons why saturations in siRNA delivery were observed. First, the cells simply could no longer uptake siRNA because of the limitations in transporter or cellular components that enabled the delivery of siRNA-lipid carrier complex. If this was the case, changing the ratio of lipid carrier to siRNA would not alter the amount of siRNA delivered. Another possible reason why saturations occurred was the existence of uncomplexed siRNA due to the lack of lipid carrier amount needed to create siRNA-lipid complex, which is further delivered into the CHO cells. It is known that uncomplexed siRNA would not be transported into the cells.

To test these two hypotheses, the Lipofectamine 2000 dose was increased to various concentrations for various amounts of siRNA used as shown in Figure 4.3. It is seen in Figure 4.4, increasing the dose of lipid carrier by 3x and 10x increased the amount of siRNA delivered for every concentrations of siRNA. At the lowest concentration of siRNA (100 pmole), the mean fluorescence increased by 48% when the Lipofectamine was increased 3-fold (from 1 μ L to 3 μ L). However, a further increase in Lipofectamine of another 3-fold (from 3 μ L to 10 μ L) only yielded a mean fluorescence increase of 10%, showing a slow down in the increasing rate of siRNA delivery. This phenomenon demonstrated that the availability of siRNA had become the limiting factors in increasing the mean fluorescence for an increasing amount of lipid carrier. Alleviating this problem was easily done by increasing the amount of siRNA delivered for a given amount of lipid carriers.

As illustrated in Figure 4.4, increasing the amount of siRNA followed by increasing amount of Lipofectamine 2000 could certainly increase the amount of siRNA transported into CHO cells because the rate limiting steps lied on the formation of siRNA-lipid complex. However, Lipofectamine was toxic to the growth of CHO cells. In fact, we found that siRNA-lipofectamine complex should only be exposed to CHO cells for about 4 hours, followed by a replacement medium consisting of basal medium supplemented by twice the amount of heat-inactivated fetal bovine serum (from 10% IFS to 20% IFS) to promote cell growth. Therefore, design of siRNA experiments using Lipofectamine 2000 should take into account the percentage of cell viability post transfection.

Many gene knock down experiments using RNA interference technique could only be identified 48-60 hours post transfection as this is the typical turnover time for existing transcript or protein. If most cells were no longer viable 48 – 60 hours post transfection due to Lipofectamine 2000 toxicity, real time RT-PCR and/or protein activity assay could not accurately identify the efficacy of a particular siRNA sequence in knocking down a gene. In fact, during our studies, experiments in numerous 6-well plates must be done to pool sufficient viable cells for RNA or protein assay. Collecting viable cells in anchorage-dependent cell cultures was relatively easy to do because the non-viable cells would no longer attach on the surface of the cell culture plate and aspirating the supernatant removed all the non-viable cells. However, collecting viable cells in suspension system could be challenging because there were no easy and accurate methods to quickly separate non-viable cells from viable cells. Therefore, in designing our experiments, anchorage-dependent cell lines were used for this initial study.

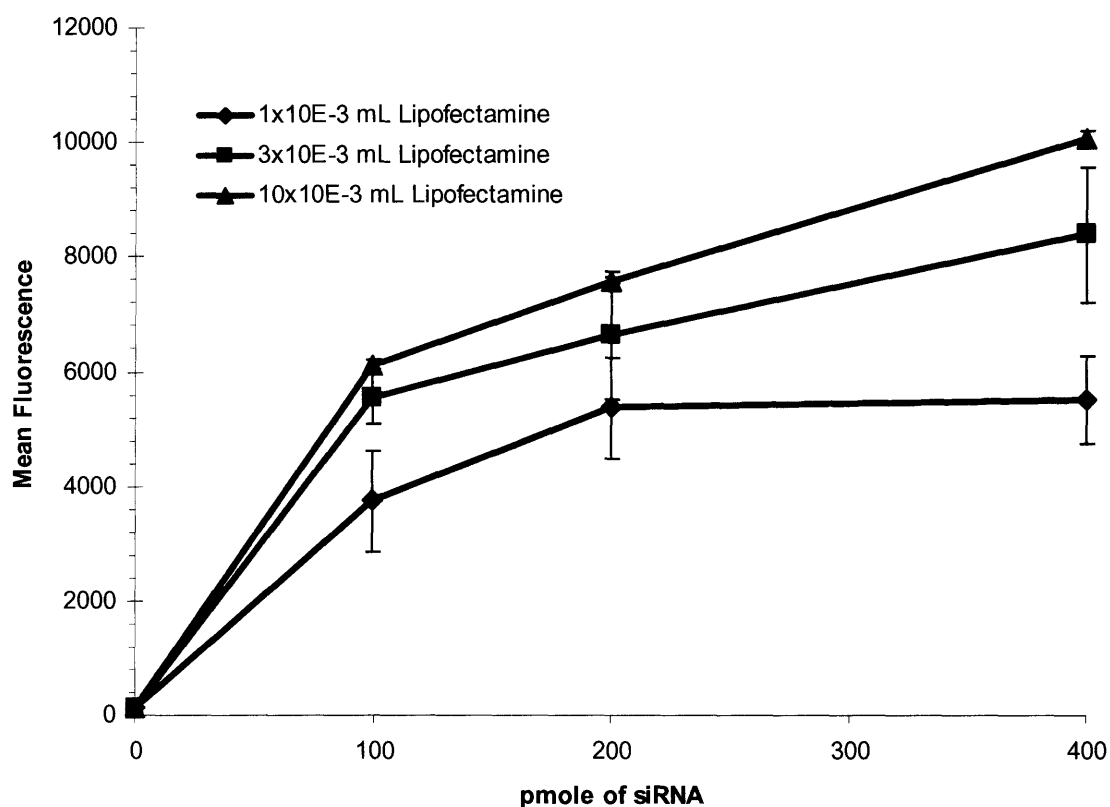


Figure 4.4 The effect of increasing the dosage of Lipofectamine 2000 transfection reagent on the delivery of fluorescein-labeled siRNA

Non-silencing fluorescein labeled siRNA was delivered utilizing different dose of Lipofectamine to assess the optimal quantity of transfection reagent. Error bars represent the range of measurements taken from four cultures of CHO-IFN γ .

Real Time RT-PCR and Protein Activity Assay to Analyze the Potency of siRNA Sequences

During the time we started this study, Tuschl's rule was the only available guidelines in designing siRNA for a particular gene. Since the guidelines are empirical, following Tuschl's rule does not guarantee a success in designing potent siRNA. In this study, we have tested ten sialidase siRNA sequences whereby five of them followed Tuschl's rule and the other five were randomly picked across the cDNA of Chinese Hamster Ovary's sialidase, ignoring Tuschl's rule. The sequences, in the form of siRNA duplex, are shown in Table 4.2.

Total RNA from cell lysate was obtained 48 hours post siRNA transfection and from quantitative RT-PCR assay we could easily quantify the amount of remaining sialidase transcript from the constant amount of RNA (1 μ g) and from the constant amount of viable cells ($0.1 - 1 \times 10^6$ viable cells). As shown in Figure 4.5, the best sequence S1, which did not follow Tuschl's rule, showed a knock down of sialidase mRNA by 9 folds. The knock down in sialidase mRNA was followed by 4-fold knock down in sialidase activity, as measured using methylumbelliferyl fluorescence substrate (Figure 4.6). The agreement between quantitative PCR assay (which measured knock down in mRNA level) and fluorescence assay (which measured knock down in enzymatic activity) was observed for nearly all sialidase siRNA sequences (Figure 4.7). It was worth noting that although the best sequence did not originate from Tuschl's rule, all five designs based on Tuschl's rule implementation yielded sialidase mRNA knock down by 2-3 folds. This indicated the generality of Tuschl's rule as a starting point to design siRNA as random selection across the sialidase cDNA besides sequence S1 (sequence S4, S8, S9, S10) only yielded ≤ 2 folds transcript knock down.

Table 4.2 Sialidase siRNA sequences designed using Tuschl's rule and arbitrary choices.

Sequences S1, S4, S8, S9, S10 were designed randomly to bind various parts of the CHO-IFN γ cytosolic sialidase 1366-nt cDNA sequences. Sequences S2, S3, S5, S6, and S7 were designed following Tom Tuschl's empirical rule

Description	Target Sequence	Tuschl's Rule?
Sialidase siRNA Sequence S1	5'AUCAUCUGCAGGGCCUCGGUU3' 3'UUUAGUAGACGUCCCGGAGCC5'	No
Sialidase siRNA Sequence S2	5'GGCUGCCAUGGAAGUGUGAUU3' 3'UUCCGACGGUACCUUCACACU5'	Yes
Sialidase siRNA Sequence S3	5'GCAGAAGACCCUGCUGGCCUU3' 3'UUCGUCUUCUGGGACGACCGG5'	Yes
Sialidase siRNA Sequence S4	5'GGAGACUAUGCUUACAGAAUU3' 3'UUCCUCUGAUACGAAUGUCUU5'	No
Sialidase siRNA Sequence S5	5'GCCGGUCCUCCCUUCUCCAUU3' 3'UUCGGCCAGGAGGGAAGAGGU5'	Yes
Sialidase siRNA Sequence S6	5'GACGGAUGAGCAUGCAGAUUU3' 3'UUCUGCCUACUCGUACGUCUA5'	Yes
Sialidase siRNA Sequence S7	5'GCAAGCUUUC CAGCAGUGUU3' 3'UUCGUUCGAAAGGUGGUCAC5'	Yes
Sialidase siRNA Sequence S8	5'GACUAUGCUUACAGAAUCCUU3' 3'UUCUGAUACGAAUGUCUUAGG5'	No
Sialidase siRNA Sequence S9	5'GAUUUGUUUGUCCUACGAAUU3' 3'UUCUAAACAAACAGGAUGCUU5'	No
Sialidase siRNA Sequence S10	5'CUCGGACUUGCAGAACAUUU3' 3'UUGAGCCUGAACGUCUUGUAC5'	No

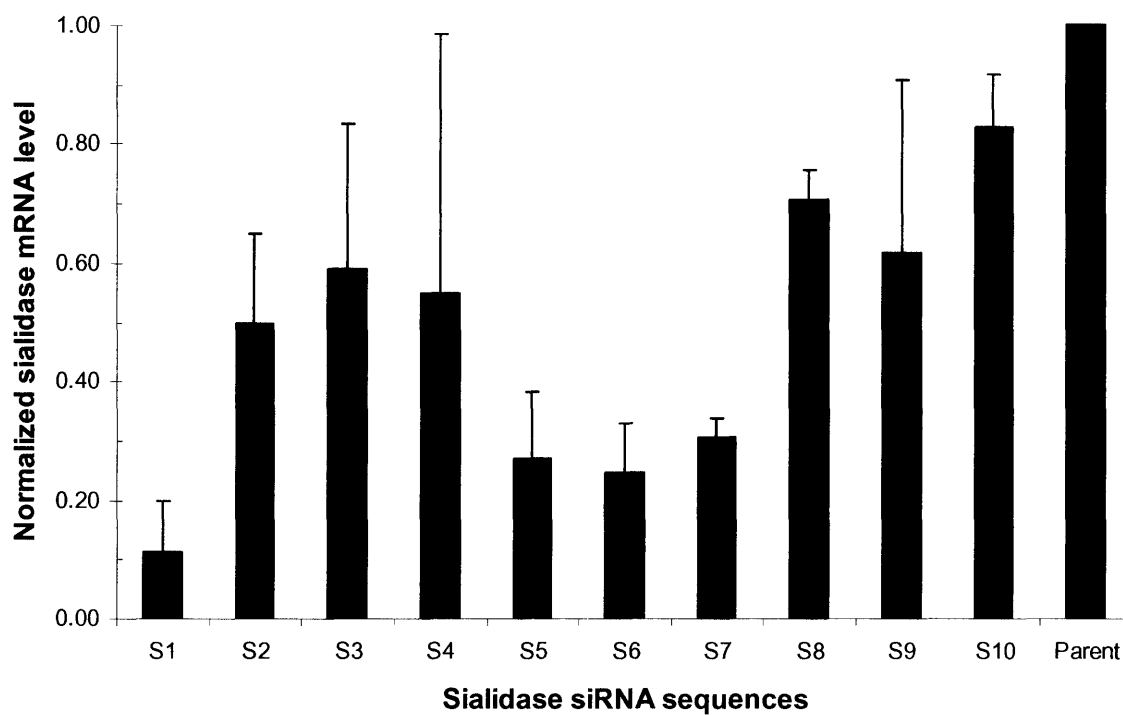


Figure 4.5 mRNA quantification of cells transfected with sialidase siRNA tabulated in Table 4.2.

Total RNA was isolated from each CHO-IFN- γ cells transfected with sialidase siRNA (depicted in Table 4.2) and two step RT-PCR was performed. β -actin mRNA was utilized as an internal control and reported sialidase mRNA level from quantitative PCR assay was normalized with β -actin mRNA level.

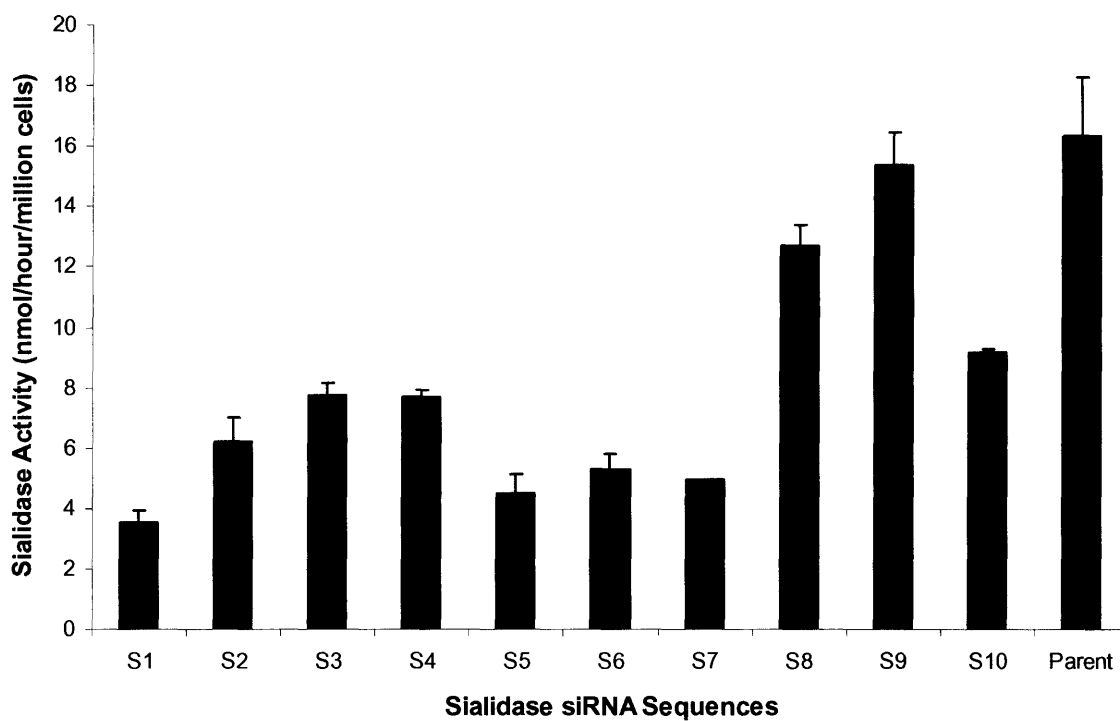


Figure 4.6 Sialidase activity of CHO-IFN γ cells transfected with sialidase siRNA tabulated in Table 4.2.

CHO-IFN γ cells transfected with sialidase siRNA were collected 48 hours post transfection. 1×10^6 cells were lysed and the lysate was subjected to fluorescence assay to measure sialidase activity.

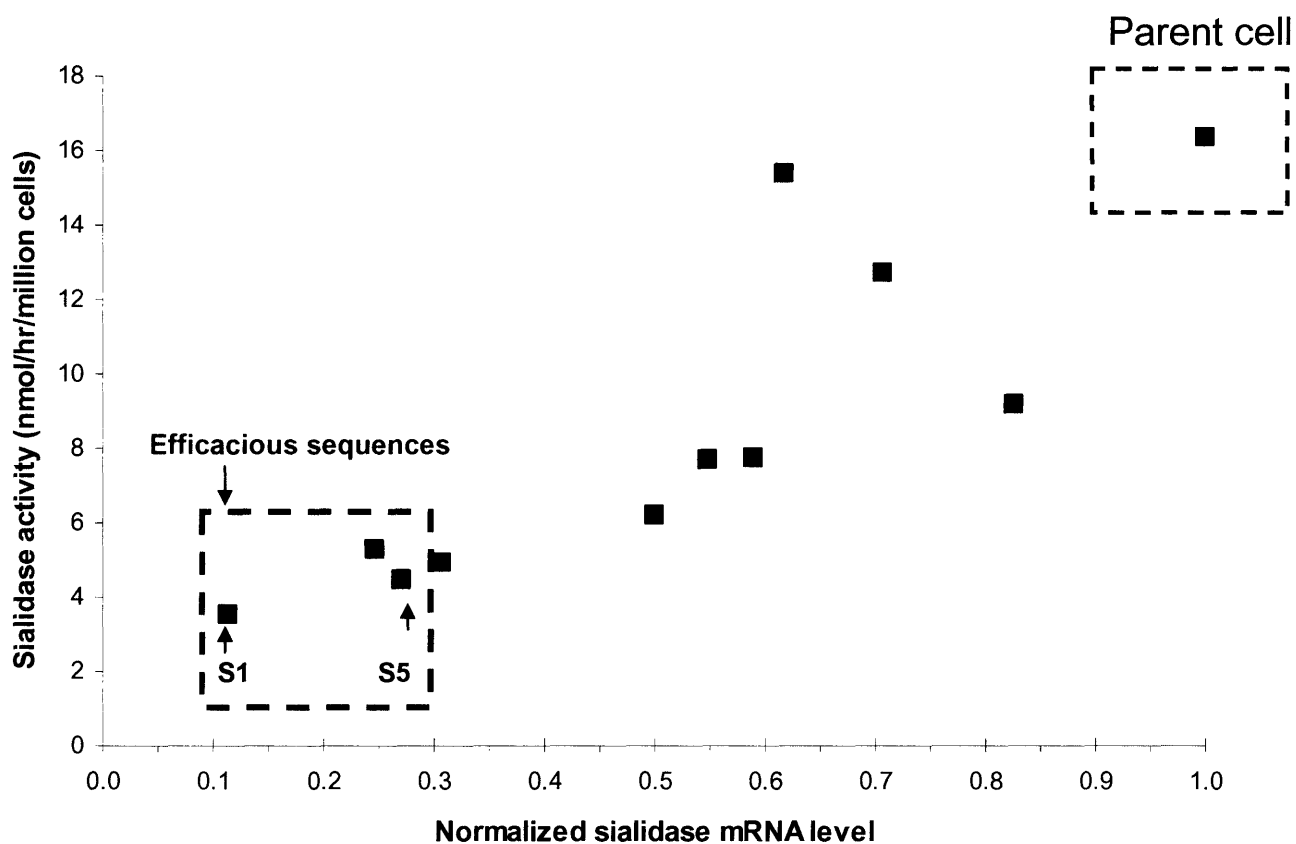


Figure 4.7 Reduction in sialidase mRNA level by double stranded RNA interference mechanism corresponds to the decrease in sialidase activity

Quantitative PCR analysis and sialidase fluorescence activity assay using methylumbelliferyl substrate were shown to be equally efficacious methods to measure sialidase activity. A decline in sialidase transcript was shown to decrease sialidase activity in the CHO-IFN γ cell lysate.

It was worth noting that over-estimate in sialidase activity resulting from 4-methylumbelliferyl fluorescence assay of cell lysate was possible. In our study, cell lysate was used as the source of sialidase measurements because serum, which was generally added into anchorage dependent cell lines as growth supplements, exhibited fluorescence within the excitation and emission range of fluorescence assay experiments. This indicated the existence of sialidase within serum, as previously observed by many other researchers. Heat-inactivating serum did not eliminate the fluorescence background, which indicated the inability to eliminate sialidase activity entirely or the existence of other components within serum that produced background fluorescence within the excitation and emission range of 4-methylumbelliferyl substrate. By using cell lysate that was washed over three times to remove residual sialidase from growth media, a better estimate of sialidase activity was expected to be obtained. However, although the objective of our study was to eliminate the soluble cytosolic sialidase responsible in cleaving sialic acid of glycoprotein in supernatant, there were many other sialidases whose activities were indiscriminately measured within cell lysate by methylumbelliferyl fluorescence assay.

For Chinese Hamster Ovary cell lines, soluble cytosolic sialidase responsible for cleaving sialic acid off glycoproteins is the only sialidase cloned to this day (Ferrari et al., 1994). Other forms of sialidase in CHO cell lines have not been characterized in details because of the relatively low abundance of sialidase in all tissues. In other mammalian systems (such as *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Cricetulus griseus*, *Bos Taurus*), there were four types of sialidase identified and cloned: lysosomal, cytosolic, and two types of plasma membrane-associated sialidases (Hasegawa et al., 2001; Monti et al., 2002). In rat liver, it was well characterized that lysosomal and cytosolic sialidases hydrolyzed glycoproteins (Miyagi et al., 1993). These

sialidases were also found to hydrolyze 4-methylumbelliferyl-N-acetylneuraminic acid, which was the fluorescence substrate used in our study. Considering the close proximity between rat and hamster genomic sequences, it was our belief that the existence of more than one sialidases was true for CHO cells. Therefore, by performing a sialidase assay in cell lysate, we estimated the activity originating from all of the sialidases. A knock down of sialidase activity by 2-fold could actually represent a knock down of cytosolic sialidase activity by 2-fold or more since the other sialidases (such as lysosomal sialidase) still cleaved fluorimetric substrate.

Thermodynamic Analysis of Sialidase siRNA Sequences

In our studies, we demonstrated that the most potent sialidase siRNA was found through random selection. Although Tuschl's rule provided choices of sequences that yielded reduction in sialidase mRNA levels, a better understanding on why sequence S1 worked better despite its violation of Tuschl's rule would lead to a better siRNA design for other applications in the future. At the time of this thesis, there have been many publications that attempt to rationalize the siRNA sequences design (Khorova et al., 2003; Schwartz et al., 2003; Amarzguioui and Prydz, 2004; Chalk et al., 2004; Reynolds et al., 2004; Ui-Tei et al., 2004). Most works attempted to come up with design criterion based on statistical analysis on published results and the success rate of this approach is limited. Only the works performed by Khorova and Schwartz have established a design rule based on acceptable thermodynamic analysis derived from an understanding of the mechanism of RNAi. In this section, we seek to understand whether the success of sequence S1 in knocking down sialidase assay was due to thermodynamic reasoning.

Schwartz' asymmetrical rule and Khvorova's internal stability analysis were inspired by the understanding that one of the critical steps in RNA interference mechanism was the recruitment of the anti-sense strand of an siRNA duplex by the RISC complex. This single-stranded antisense further guided the complex to the messenger RNA that possessed the complementary sequence, which resulted in the endonucleolytic degradation of target mRNA (Dykxhoorn et al., 2003). Thus, it was critical that the siRNA duplex unwound in a way that enabled anti-sense strand of siRNA to be recruited by RISC.

Schwartz and her co-workers argued that RISC assembly favored the siRNA strand whose 5' end was weaker in terms of binding energy. In particular, the binding energy in the antisense 5' end must be weaker as to allow helicase to initiate unwinding process from 5' end of antisense strand, leading to the assembly of single stranded antisense strand to RISC complex. A recruitment of sense strand into RISC complex was thought to produce no RNA interference process. This was illustrated in Figure 4.8 (adapted from Schwartz et al., 2003).

Free energy of the sense strand of the siRNA (ΔG_{sense}) and free energy of the antisense strand of the siRNA ($\Delta G_{\text{antisense}}$) were calculated using nearest-neighbor method and the mfold algorithm (Zuker, 2003; Ding et al., 2004). Their difference was called $\Delta\Delta G_{\text{Sense-Antisense}}$ whereby instability in 5' antisense end would correspond to a $\Delta\Delta G$ value of less than zero. The result of the estimate was tabulated in Table 4.3 and the sialidase activity from experimental result was compared to the $\Delta\Delta G$ estimated ala Schwartz' et al. method (Figure 4.9). Although $\Delta\Delta G < 0$ trend was observed for some functional sialidase siRNA sequences, there was no strong correlation between the negativity of $\Delta\Delta G$ values and the siRNA efficacy. In fact, sequence S1, which was

the best sequence found in our study, possessed positive $\Delta\Delta G$ values, indicating the fact that Schwartz' asymmetry rule would not suggest the utilization of sequence S1 in knocking down sialidase activity. Obviously, the success in sequence S1 efficacy was not explained by a weaker 5' antisense strand.

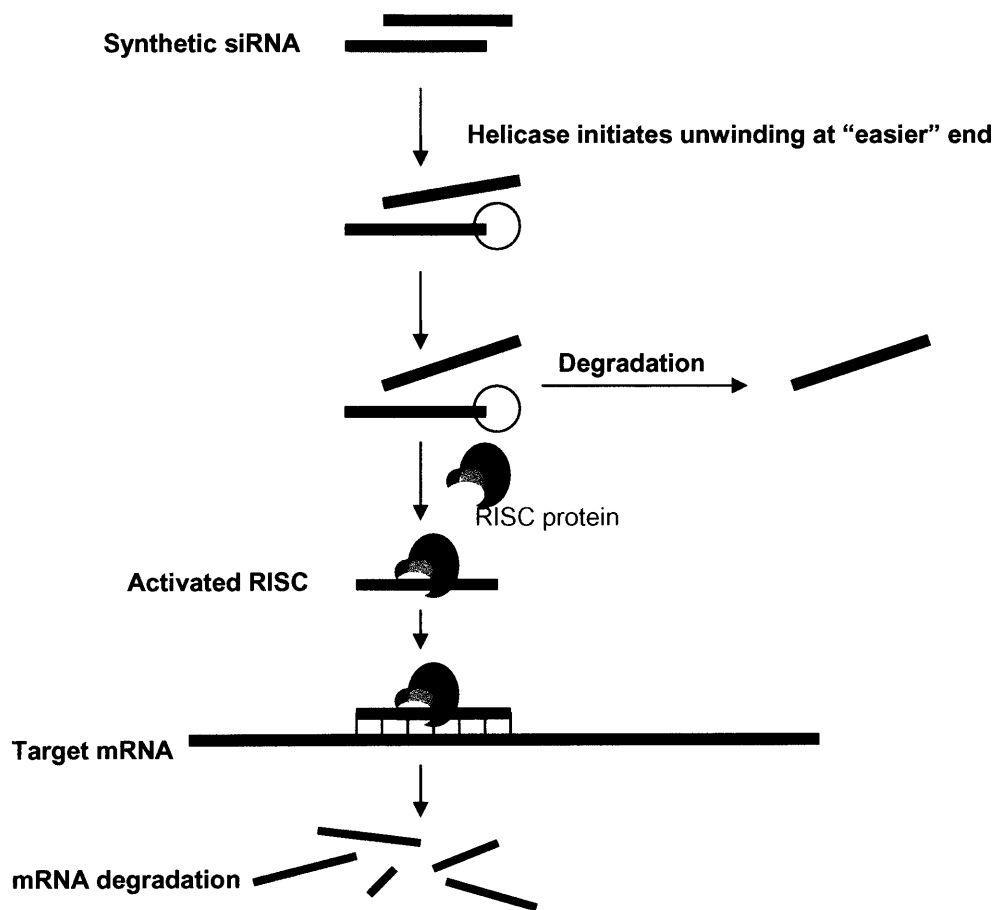


Figure 4.8 Model for RISC assembly based on Schwartz' asymmetry rule. This figure is adapted from Schwartz et al. (2003).

Table 4.3 Thermodynamic analysis of various sialidase siRNA sequences

The stability of the initial four base pairs of siRNA antisense sequence ($\Delta G_{\text{Antisense}}$) was calculated using the nearest-neighbor method and the mfold algorithm (Mathews et al., 1999, Zuker, 2003). The same calculation was done for the siRNA sense sequence (ΔG_{Sense}). Based on Schwartz et al. asymmetrical rule analysis, $\Delta\Delta G = \Delta G_{\text{Sense}} - \Delta G_{\text{Antisense}} < 0$ is desired (Schwartz et al., 2003). The average internal stability values for positions 9-14 of the antisense strand (AIS) were calculated as demonstrated by Khvorova et al. (Khvorova et al., 2003). AIS > -8.5 kcal/mol strongly correlated to potency of siRNA design.

Sequence	$\Delta G_{\text{Antisense}}$ (kcal/mol)	ΔG_{Sense} (kcal/mol)	$\Delta\Delta G_{\text{Sense-Antisense}}$ (kcal/mol)	AIS (kcal/mol)
S1	-8.4	-8.3	0.1	-9.8
S2	-6.1	-11.5	-5.4	-9.3
S3	-9.3	-7.1	2.2	-9.7
S4	-4.9	-8.4	-3.5	-7.7
S5	-7.0	-10.9	-3.9	-10.8
S6	-4.9	-9.8	-4.9	-8.9
S7	-7.0	-8.1	-1.1	-8.4
S8	-8.3	-7.2	1.1	-7.6
S9	-4.9	-6.2	-1.3	-7.0
S10	-5.9	-10.9	-5.0	-8.6

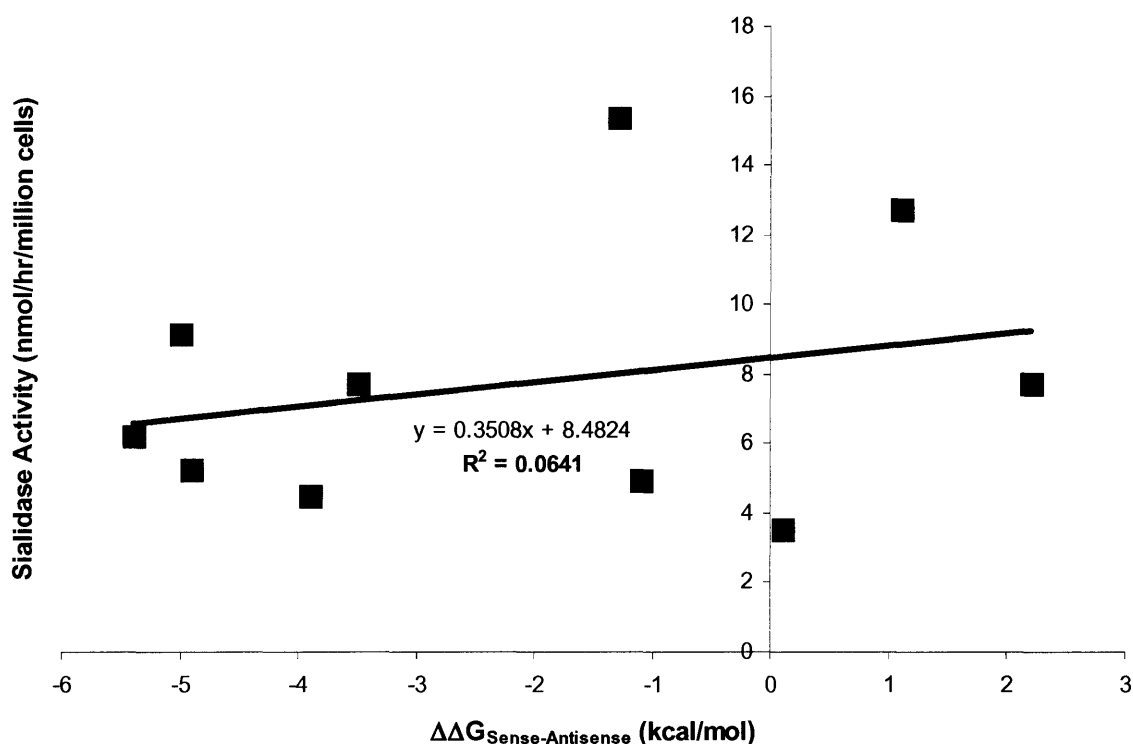


Figure 4.9 Thermodynamic analysis utilizing Schwartz et al.'s asymmetrical rule did not adequately predict reduction in sialidase activity contributed from small interfering RNA

The black squares (■) denote sialidase activity measured utilizing fluorescence assay compared to asymmetrical thermodynamic parameters estimated per Schwartz et al. method. Following the Schwartz asymmetrical rule, sequences with $\Delta\Delta G < 0$ should silence better than those with $\Delta\Delta G > 0$. Although this trend was somewhat observed for a few sequences, this methodology suffered from false positive and false negative phenomenon. This indicated that asymmetrical rule was not a perfect predictor of the sialidase siRNA sequences efficacy in knocking down sialidase.

Another approach to understand the reason behind the potency of S1 sequence was to employ Khvorova's (2003) average of internal stability analysis for positions 9 – 14 of the antisense strand (AIS) analysis. A less stable antisense strand was desired to allow initiation of helicase at 5' antisense strand end and this would be represented by more positive AIS value. A rule of thumb established by Khvorova's et al. was that the AIS at positions 9-14 should be lower than – 8.5 kcal/mol. This analysis was conducted on the sequences displayed in Table 4.2. We found that our experimental data exhibited the opposite behavior than the ideal behavior established by Khvorova et al. (Figure 4.10). In fact, sialidase siRNA sequence S1 had the most negative AIS values, an indication of stable antisense strand at positions 9 -14. We could not conclude that thermodynamic analysis was an ineffective tool, but we could certainly conclude that the best sialidase siRNA sequence potency did not originate from having an instable antisense strand as promoted by Khvorova et al.

It is interesting to note that sialidase siRNA sequence S1 was not in the open reading frame (ORF) region of sialidase cDNA. In fact, sequence S1 was a part of 5' untranslated region (UTR). It was hypothesized by Elbashir and her co-workers that UTR binding proteins might interfere with scanning process of the siRNA complex and thus, 5' and 3' UTR should be avoided when designing efficacious siRNA. These empirical rules were refuted by many recent works. Yokota and his co-workers in Japan successfully knocked down HCV replication by 80% when targeting the 5' UTR region with an siRNA dose as low as 2.5 nM. (Yokota et al., 2003). Targeting the 3'UTR region using siRNA was also found to be effective as demonstrated by a recent work in knocking down CD4 and CD8 α in murine thymocyte cell lines (McManus et al., 2002b).

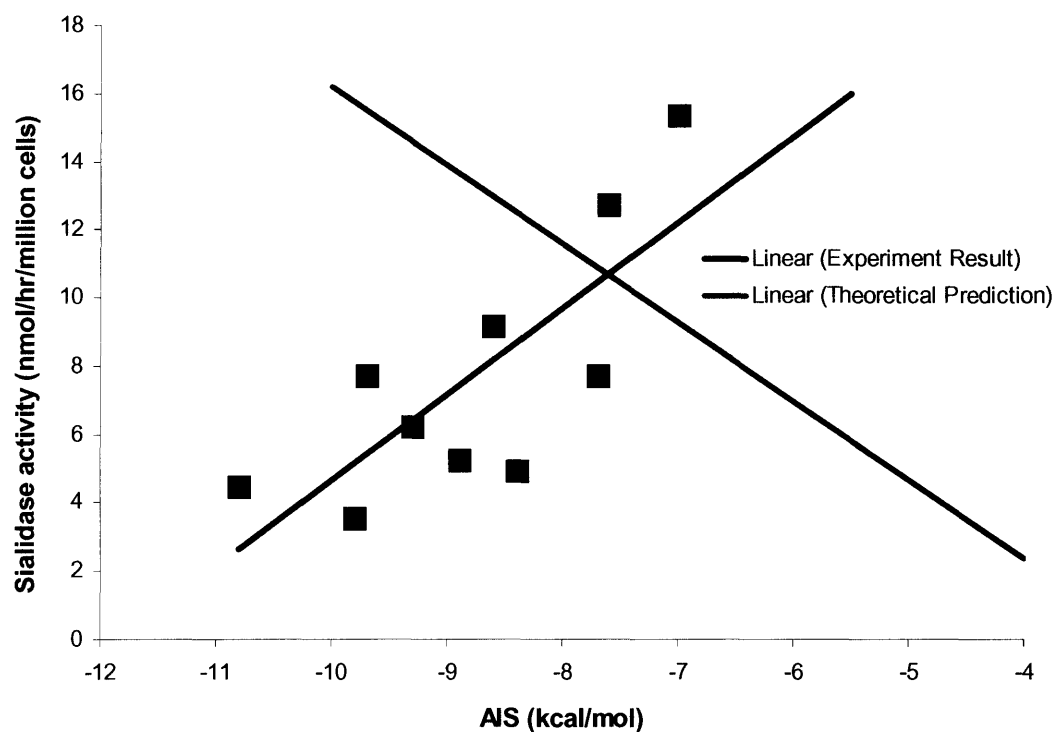


Figure 4.10 Thermodynamic analysis utilizing Khvorova's average of internal stability analysis for positions 9-14 of the antisense strand (AIS) prediction yielded the opposite trend shown by experimental results.

Average of internal stability values for positions 9-14 of the antisense strand (AIS) was calculated as demonstrated by Khvorova et al. (2003). Based on their analysis, it was predicted that a double stranded RNA bearing less negative AIS should yield a more potent silencing effect. In our experiment, we observed the opposite trend.

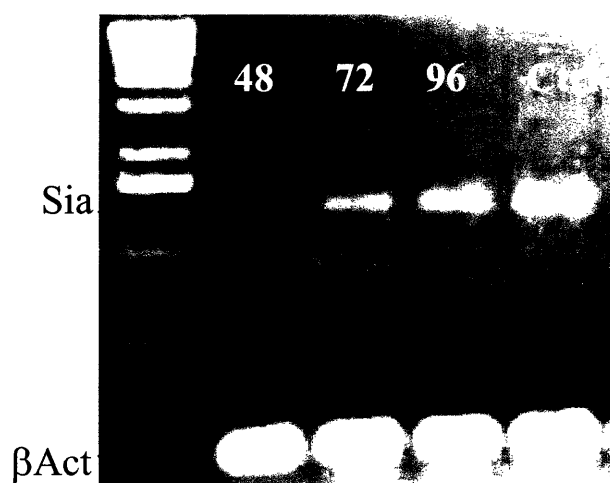
In summary, there was not a scientific explanation of why a certain siRNA sequence worked better than others in knocking down a specific gene expression. Tuschl's rule, thermodynamic analysis, and many other empirical rules should only be used as guidelines, although searching sequences randomly across various location of the cDNA (including UTRs) was still the best method to locate an efficacious siRNA sequence.

siRNA Transiently Induce Sialidase Silencing in Various CHO Cell Lines

In order to make siRNA technique a general method to prevent desialylation action by cytosolic sialidase, it was essential to identify the duration of silencing given by siRNA method and to investigate whether continuous silencing was possible. A simple experiment using three of the efficacious sialidase siRNA was performed over 144 hours post-transfection culture times to observe the extent of gene knock down by sialidase siRNA. As shown in Figures 4.11 – 4.14, 48-hours after sialidase siRNA transfection, Sequence S1, S5, and 56 successfully decreased sialidase mRNA levels. This effect was only transient as sialidase activity gradually increased to its initial level after 72 hours. RT-PCR assays and activity assays yielded similar trends in detecting transient sialidase reduction. This transient behavior occurs because there was a limited amount of siRNA initially introduced into the supernatant. Once this initial siRNA amount was exhausted, more siRNA is required to retain silencing. It was worth noting that sialidase assayed for activity in this study was obtained from cell lysate.

Activity assays can also be done on sialidase concentrated from supernatant. Performing activity assay on cell lysate was deemed to be a more rigorous approach as the sialidase in supernatant originated from lysate in any case. In addition, fetal bovine serum (in which the exact

Lane 1 2 3 4 5



Lane 1: DNA ladder

Lane 2: PCR product of sialidase cDNA reverse transcribed from RNA of siRNA-transfected cells. The RNA was obtained 48 hours post transfection of sialidase siRNA

Lane 3: PCR product of sialidase cDNA reverse transcribed from RNA of siRNA-transfected cells. The RNA was obtained 72 hours post transfection of sialidase siRNA

Lane 4: PCR product of sialidase cDNA reverse transcribed from RNA of siRNA-transfected cells. The RNA was obtained 96 hours post transfection of sialidase siRNA

Lane 5: PCR product of sialidase cDNA reverse transcribed from RNA of normal cells untransfected with sialidase siRNA.

Figure 4.11 RT-PCR analysis of interfering CHO-IFN γ sialidase mRNA using siRNA sequence S1

IFN γ -producing CHO cells were transfected with siRNA Sequence S1 whose DNA sequences are shown in Table 1. RNA was isolated using TRIzol reagent and used for RT-PCR. As shown here, 48-hours post transfection, sialidase siRNA successfully reduced sialidase mRNA levels. This effect was only transient as sialidase activity gradually increased to its initial level after 72 hours.

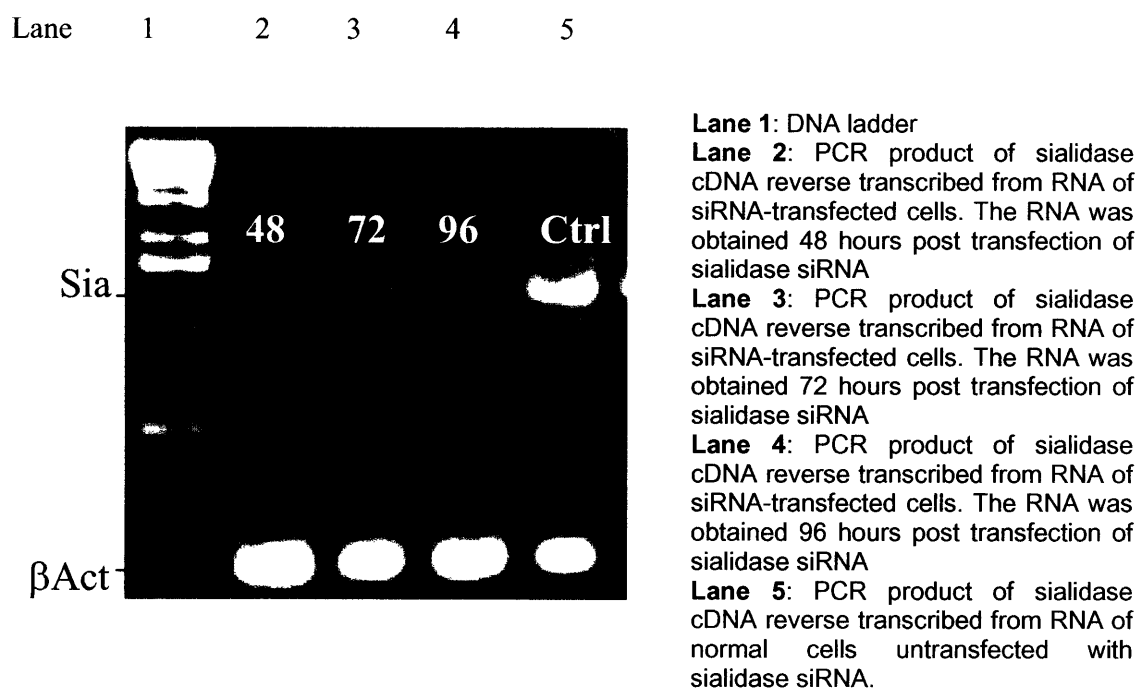


Figure 4.12 RT-PCR analysis of interfering CHO-IFN γ sialidase mRNA using siRNA sequence S5

IFN γ -producing CHO cells were transfected with siRNA Sequence S5 whose DNA sequences are shown in Table 1. RNA was isolated using TRIzol reagent and used for RT-PCR. As shown here, 48-hours post transfection, sialidase siRNA successfully reduced sialidase mRNA levels. This effect was only transient as sialidase activity gradually increased to its initial level after 120 hours (data now shown).

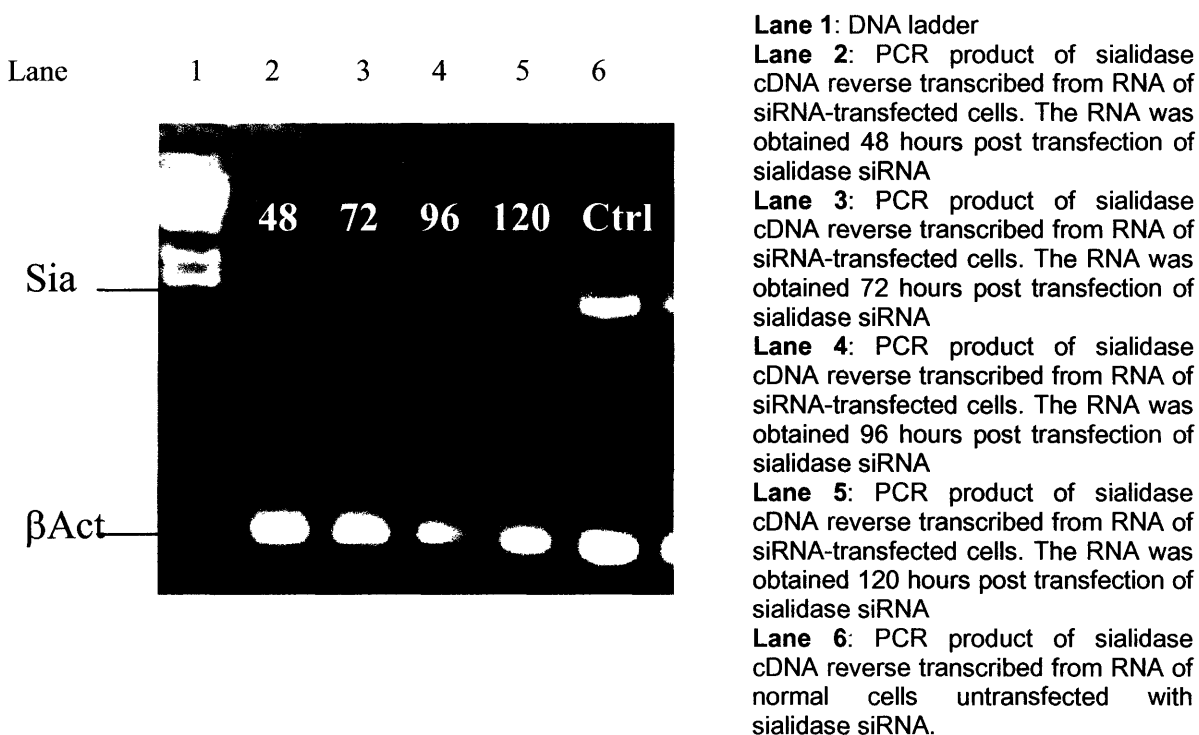


Figure 4.13 RT-PCR analysis of interfering CHO-IFN γ sialidase mRNA using siRNA sequence S6

IFN γ -producing CHO cells were transfected with siRNA Sequence S6 whose DNA sequences are shown in Table 1. RNA was isolated using TRIzol reagent and used for RT-PCR. As shown here, 48-hours post transfection, sialidase siRNA successfully reduced sialidase mRNA levels. This effect was only transient as sialidase activity gradually increased after 72 hours.

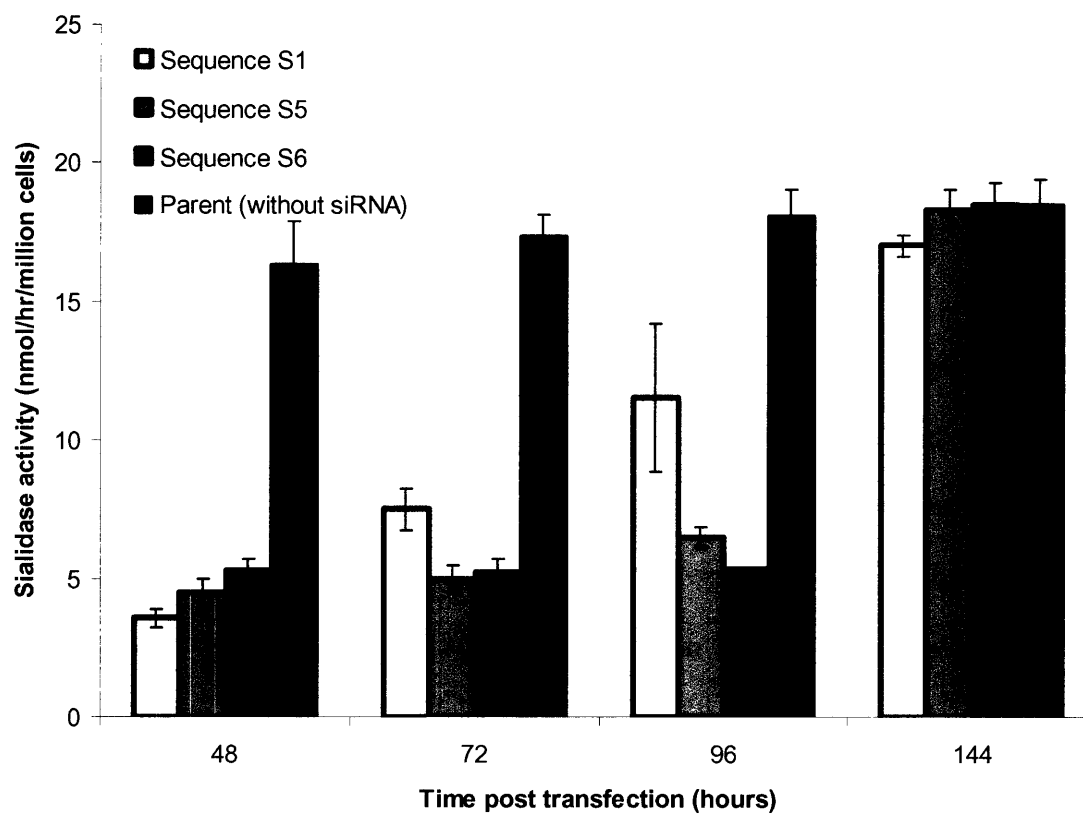


Figure 4.14 Sialidase activity reduction as a result of transient CHO-IFN γ sialidase mRNA silencing using siRNA sequence S1, S5, and S6.

This activity assay reconfirmed the transient nature of siRNA silencing shown in Figure 1. There were no discrepancies between the RT-PCR experiment, which measured sialidase mRNA levels and the fluorescence activity assay, which measured sialidase protein activity levels. As shown here, 48-hours post transfection, sialidase siRNA successfully reduced sialidase mRNA levels. This effect was only transient as sialidase activity gradually increased to its initial level (at 144 hours).

composition varied from lot to lot) added in supernatant tended to contribute background fluorescence, hence, biasing the measurements of sialidase activity. Even in the transformed suspension culture absent of serum, from which sialidase was isolated and concentrated using a Microsep concentrator with 10kDA exclusion limit, the HyQ PF-CHO medium contributed high fluorescent background making the activity measurement unreliable. The difficulty in measuring sialidase in supernatant was also observed by other researcher (Sung et al., 2004). Therefore, we did not perform sialidase assays on supernatant.

To investigate whether continuous silencing of siRNA was possible, sialidase sequence S1 was re-transfected at the point when sialidase activity reverted to its normal level. The same dose of siRNA was utilized and the same amount of CHO cells (0.1 million cells) were transfected to ensure similar experiment conditions. It was observed that 48 hour post retransfection, sialidase activity reduced significantly with magnitudes of reduction similar to the first transfection process (Figure 4.15). However, this second transfection process also exhibited transient silencing because sialidase level reverted back to its untransfected level five days post retransfection. This simple study demonstrated that transient nature of siRNA method could be overcome by continuously dosing the cells with appropriate amount of siRNA. In the future, a genetic method to continuously produce sialidase siRNA would become the ideal platform for industrially-relevant cell culture applications.

Utilization of sialidase siRNA as a method to increase sialic acid content should be universal to any CHO cells. This is because CHO, with its glycosylation capability, is still the workhorse for most therapeutic glycoprotein productions. In our study, in addition to using CHO-IFN γ , we also

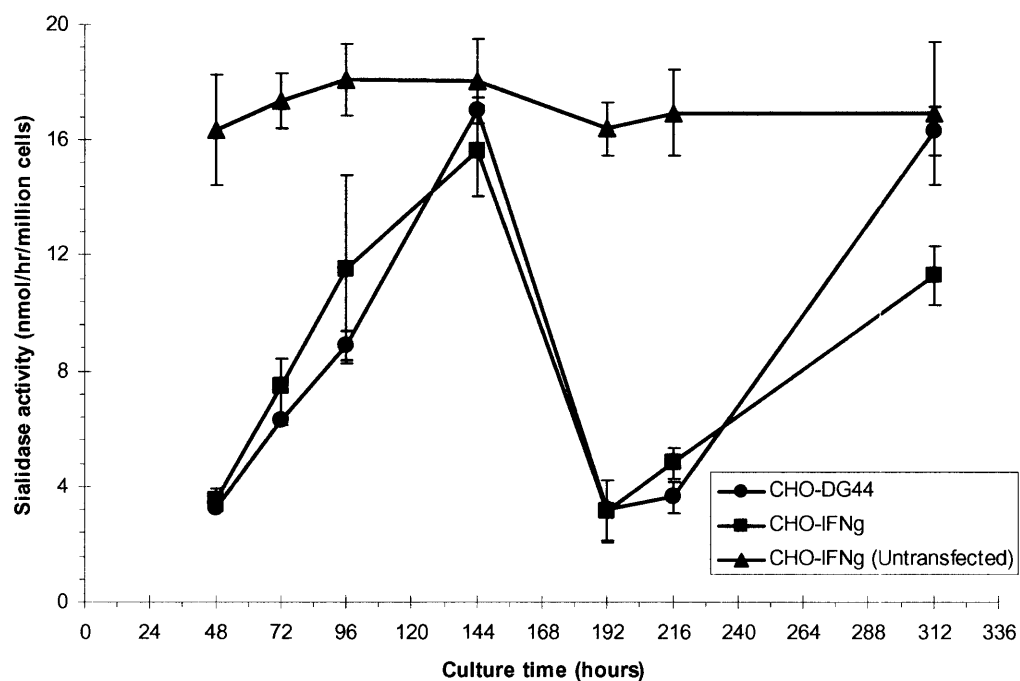


Figure 4.15 Retransfection of CHO cells with sialidase siRNA recovered the siRNA activity to reduce sialidase activity in various CHO cells.

Sialidase siRNA sequence S1 was transfected to CHO-DG44 and CHO-IFN γ at culture times $t = 0$ hours. 1×10^5 cells were collected for sialidase activity assay every 48 hours. At culture times $t = 144$ hours, each cell line was re-transfected with 5 nmol of sialidase siRNA sequence S1 and 1×10^5 cells were collected for sialidase activity assay every 48 hours. It was demonstrated that transient nature of sialidase interference via RNAi mechanism can be overcome by continuous delivery of chemically synthesized siRNA.

utilized CHO-DG44. CHO-DG44 was also used as a model because the success in knocking down a cell line that was ready to be co-transfected with any genes of interest should demonstrate the universality of siRNA approach for “blank” cell lines. As shown in Figure 4.16, transfection of sialidase siRNA sequence S1 into each CHO-DG44 and CHO-IFN γ successfully reduced sialidase activity by 5-6 folds up to 48 hours post transfection. Within 72 hours post transfection, the transient nature of sialidase transfection was observed as the sialidase activity reduction was only about 2-3 folds. As culture time increased, the dilution effect was observed again, indicating the need for continuous siRNA dosing.

4.3 Conclusions

Preliminary *in vitro* sialidase kinetics analysis predicted the severity of sialidase activity once released in the culture supernatant as the cells lost their viabilities. With increasing sialidase activity in cell culture, sialic acid would be cleaved off from the glycans on the glycoproteins and these asialoglycoproteins would have reduced biological half-life time. This phenomenon imposed the limit on production time if glycoprotein quality must be maintained, as prolonged culture time resulted in more protein with compromised pharmacokinetics behavior.

Genetic engineering of cells using RNA interference technique was proposed as a novel method to knock down sialidase activity. This should lead to a more consistent sialylated glycoprotein profile even when the culture was run for a longer time. siRNA transfection was optimized for CHO cells applications and over ten sialidase siRNA sequences were tested. One sequence was found to knock down sialidase mRNA by 9 folds and this led to a 4-fold knock down in sialidase

activity. Transfecting chemically synthesized siRNA was very effective transiently, but a stable system must be developed in the future to allow for the use of a sialidase siRNA technique for industrial relevant applications.

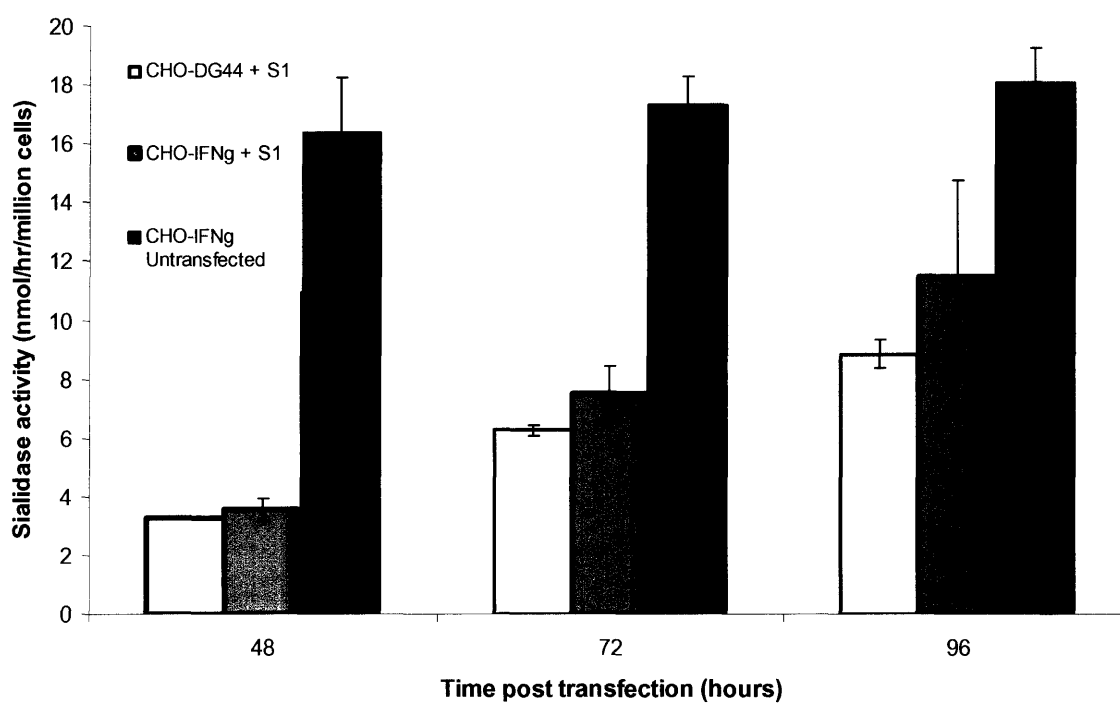


Figure 4.16 Interfering sialidase mRNA to knock down sialidase mRNA and to reduce sialidase activity is universal to various CHO cells.

Two different Chinese Hamster Ovary cells were transfected with sialidase siRNA sequence S1. The cell lysate was assayed using fluorescence substrate at different times post transfection.

5. RNA Interference of Sialidase Improves Glycoprotein Sialic Acid Content Consistency

5.1 Abstract

One of the key problems facing therapeutic protein production in mammalian cell culture is the cleavage of terminal sialic acids on recombinant protein glycans by the glycosidase enzymes released by dying cells into the supernatant. This undesired phenomenon results in a protein product which is rapidly cleared from the plasma by asialoglycoprotein receptors in the liver. In this study, RNA interference is utilized as a genetic approach to silence the activity of sialidase, a glycosidase responsible for cleaving terminal sialic acids on IFN- γ produced by Chinese Hamster Ovary cells (CHO). In the previous chapter, we have successfully identified a few 21-nt double stranded siRNA that efficiently silences endogenous sialidase mRNA and activity levels. Potency of each siRNA sequences is compared using RT-PCR and fluorescence activity assay. In this chapter, we integrated the siRNA sequence into CHO cells using a plasmid encoding a drug selection marker, allowing production and selection of stable cell lines. We have isolated stable clones with sialidase activity reduced by over 60% as compared to the control cell line. Micellar Electrokinetic Chromatography (MEKC), Thiobarbituric Acid Assay (TAA), and microheterogeneity experiments were performed to analyze glycan structures and sialic acid content. Two of the stable clones successfully retained the consistently high sialic acid content, even upon cells death. This result is comparable to the case where a chemically synthesized sialidase inhibitor was used. These results demonstrate that RNA interference of sialidase is a method that can be adopted to prevent the desialylation problem in glycoprotein production, resulting in a higher and more consistent protein quality over the life of the cell culture.

5.2 Introduction

Glycosylation is a post-translational modification processes that can only be performed properly by eukaryotic cells (reviewed in Kornfeld and Kornfeld, 1985). It is the process of adding a variety of functional carbohydrate groups to the backbone of a peptide through certain amino acid consensus sequences. Proteins that contain these sugar groups are called glycoproteins and they exist in heterogeneous populations called glycoforms. The presence of carbohydrates on the backbone of polypeptides has been shown to affect many protein properties such as: solubility, stability, biological activity, immunogenicity, and pharmacokinetics (reviewed in Jenkins and Curling, 1994).

Of numerous sugars found in glycoprotein, the most important one is the terminal sialic acid. Sialic acid is electronegatively charged acidic 9-carbon monosaccharide and is α -glycosidically linked to different positions of other sugars, most frequently to galactose or N-acetylgalactosamine and very rarely to N-acetylglucosamine or sialic acid itself (reviewed in Schauer, 1982; Varki, 1993; Angata and Schauer, 2002). Terminal sialic acid has been known to serve as a biological mask. When terminal sialic acid was removed from the glycans, desialylated serum glycoproteins have significantly lower survival times in the circulation as compared to the sialylated counterparts (reviewed in Ashwell and Harford, 1982). Sialidase treatment of glycoproteins exposes the galactose residues of glycoproteins. Then, galactose-specific lectin on hepatocytes recognizes and binds the asialoglycoproteins for degradation.

In vivo pharmacokinetics studies have been performed on glycoproteins with or without terminal sialic acid. For blood clotting Factor IX, removal of sialic acid resulted in the loss of clotting

activities at the same time courses (Chavin and Weidner, 1984). For most other glycoproteins, the removal of sialic acid correlated with significantly faster serum clearance rates and lower biological activity. Changes in circulatory half life time after desialylation for selected glycoproteins have been highlighted in Chapter 2.

The content of sialic acid in glycoproteins is affected by two opposing sialylation processes. The first process is the intracellular additions of sialic acid by sialyltransferase activities, and the second process is the extracellular removal of sialic acid by sialidase cleavage. There are many sugars, enzymes, and co-substrates involved in the metabolism of sialic acid (Reviewed in, Schauer, 2004). Cell culture conditions and genetic engineering affecting any of these components have been shown to yield an improvement or impairment in sialic acid content of glycoproteins (Gu and Wang, 1998; Fukuta et al., 2000; Bragonzi et al., 2001). Nevertheless, any attempt to increase sialic acid intracellularly must be accompanied by prevention of degradative enzyme activity because high sialic acid containing glycoprotein is still subject to desialylation by sialidase during prolonged cell culture.

In mammalian cell culture, soluble cytosolic sialidase has been isolated and characterized from culture fluid of Chinese hamster ovary (CHO) cells (Warner et al., 1993). It was found that soluble cytosolic sialidase had optimal pH near 5.5 with retention of about 50% maximal activity at typical culture pH of 7.5 (Gramer and Goochee, 1993). Accumulation of cytosolic sialidase is a general phenomenon that is observed in many other industrial-relevant cell lines albeit with different pH-activity profiles (Gramer and Goochee, 1994). As demonstrated by Gramer and Goochee (1993), an increase of lactate dehydrogenase (LDH), which signified an increase in the

cell lysis, correlated to proportional increase of extracellular sialidase activity in CHO perfusion cultures.

Reducing sialidase activity utilizing chemically synthesized inhibitor was shown to be effective in maintaining sialic acid content throughout cell culture (Gramer et al. 1995; Gu et al., 1997). However, this approach is economically justified practice only if other means to reduce sialidase cannot be practiced for industrially relevant process. A more generic technique would be to genetically inactivate cytosolic sialidase activity before it is released to the culture medium. Conventional gene knock out in mammalian cells is difficult to perform because both alleles must be targeted in order to obtain complete gene inhibition. Furthermore, even if a complete gene knock out is achieved, the effect can be deleterious if the gene product affects cell viability and metabolism (Zimmermann et al., 2002). Recent advances in RNA interference (RNAi) have made it possible to rapidly perform and study the effect of gene knock-down on a variety of cell lines (Dykxhoorn, et al. 2003; Novina and Sharp, 2004). Initially, chemically synthesized small interfering RNA (siRNA) found limited application in cell culture applications because of the transient nature of siRNA silencing. However, this is no longer true because stable silencing via RNAi mechanism is now possible by constructing vectors driven by Pol II and Pol III promoters to stably express siRNA (Brummelkamp et al., 2002; Xia et al., 2002).

In the previous chapter, various 21-nt sialidase siRNA sequences have been identified and ranked based on the potency to reduce sialidase mRNA level and to knock down sialidase desialylation activity. Chemically synthesized siRNA was transient by nature and the benefit of utilizing siRNA diminished for large scale cell cultures mainly due to the potential increased cost

using such exogenous addition of such reagents. Therefore, creating a stable cell line that produced its own sialidase siRNA would be the ideal platform for producing cells with reduced sialidase activity. Methodology to translate sialidase siRNA into stable system is highlighted in Figure 5.1. Once a stable cell line was obtained, macroheterogeneity and sialic acid content of interferon-gamma are analyzed utilizing micellar electrokinetic chromatography and thiobarbituric acid assay (Figure 5.2). A detailed microheterogeneity analysis on the sugar composition of each glycan is performed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and MALDI/TOF mass spectrometry (MS) (Figure 5.3). Macroheterogeneity and microheterogeneity analysis were conducted in collaboration with Bioprocessing Technology Institute (BTI) in Singapore.

Once a stable cell line with reduced sialidase expression is developed, the next step is to develop a rapid methodology to generate such a cell line. The general method to produce consistently high sialic acid glycoprotein that we proposed consists of three steps: transfection of potent sialidase siRNA plasmid, selection of stable cell lines, and characterization of single cell clones (Figure 5.1). Selection of single cell clones expressing a high level of a transfected gene, such as sialidase short hairpin RNA (shRNA) plasmid, is frequently the most time consuming, and thus the rate-limiting step of a process to produce stable genetically modified cell line (Zahn-Zabal et al., 2001). In addition, clonal variation is often encountered during the process of stable cell line selection (Fussenegger et al., 1999). As a result, although an individual clone strongly expresses sialidase shRNA, clonal variation might result in population with heterogeneous shRNA expression from generation to generation.

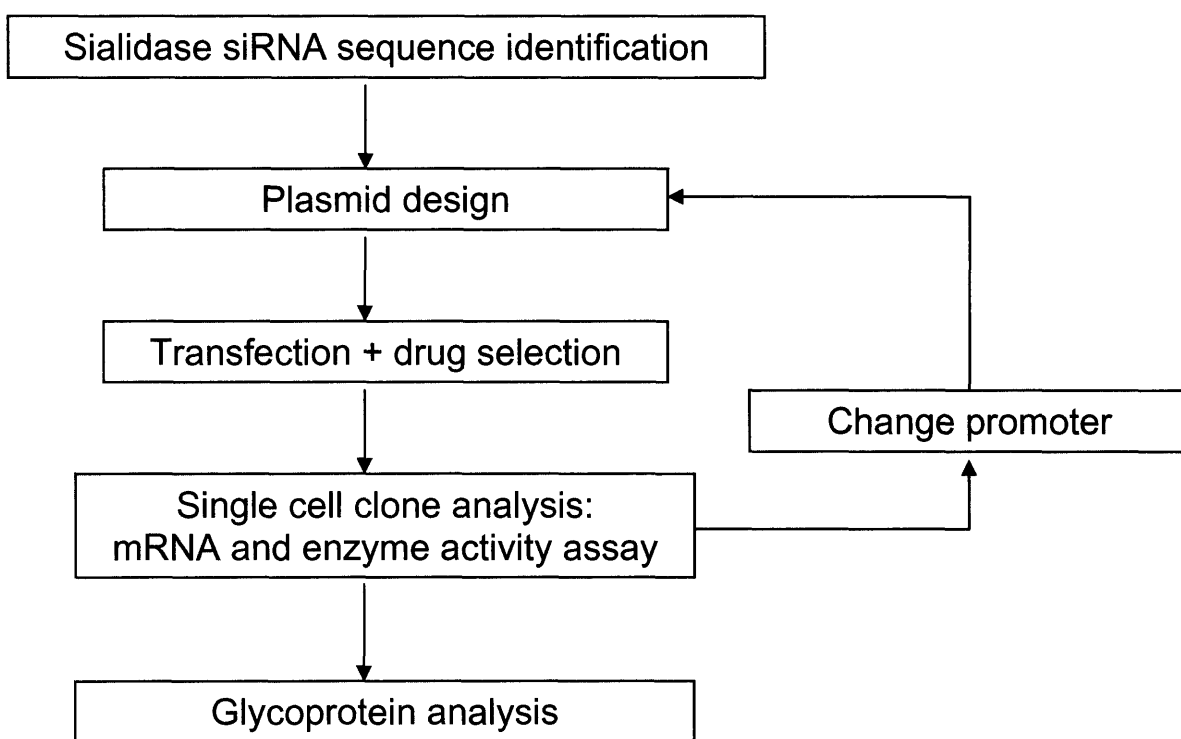


Figure 5.1 Translation of sialidase siRNA sequence from transient to stable system.

Efficacious sialidase siRNA sequence identified from transient experiment was integrated as a part of hairpin oligonucleotide. This hairpin oligonucleotide was annealed to pol II or pol III driven plasmid before the whole construct was transfected into CHO cells. Single cell clones were isolated and tested for reduction in sialidase activity. siRNA promoter could be changed to a stronger one when necessary. Once a single cell clone with reduced sialidase activity was isolated, large scale culture was performed to obtain large quantity of glycoprotein for sialylation analysis.

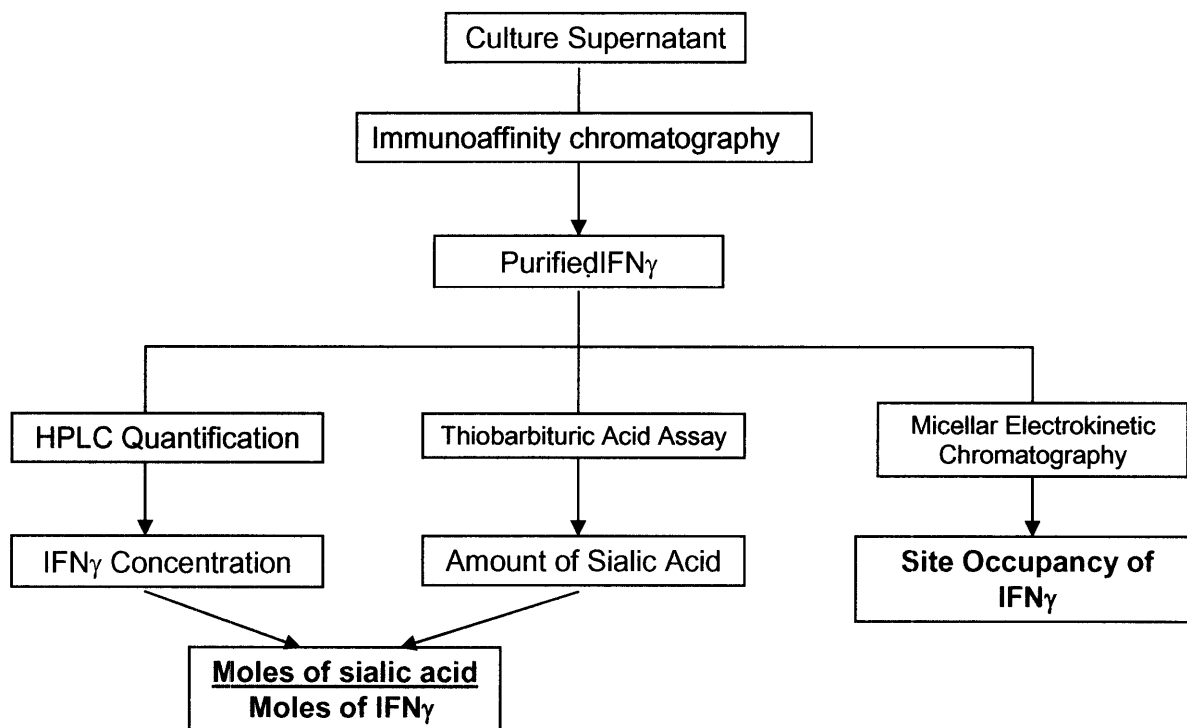


Figure 5.2 Macroheterogeneity and sialic acid quantification analysis of purified interferon gamma obtained from parent cells and single cell clones transfected with stable sialidase siRNA system.

Single cell clones with stable sialidase siRNA system were expanded into a larger culture for detailed characterization. Knocking down sialidase activity should theoretically lead to an improved sialic acid content. Also, site occupancy of interferon gamma was also analyzed to confirm that sialidase was not involved in modulation of glycoprotein site occupancy.

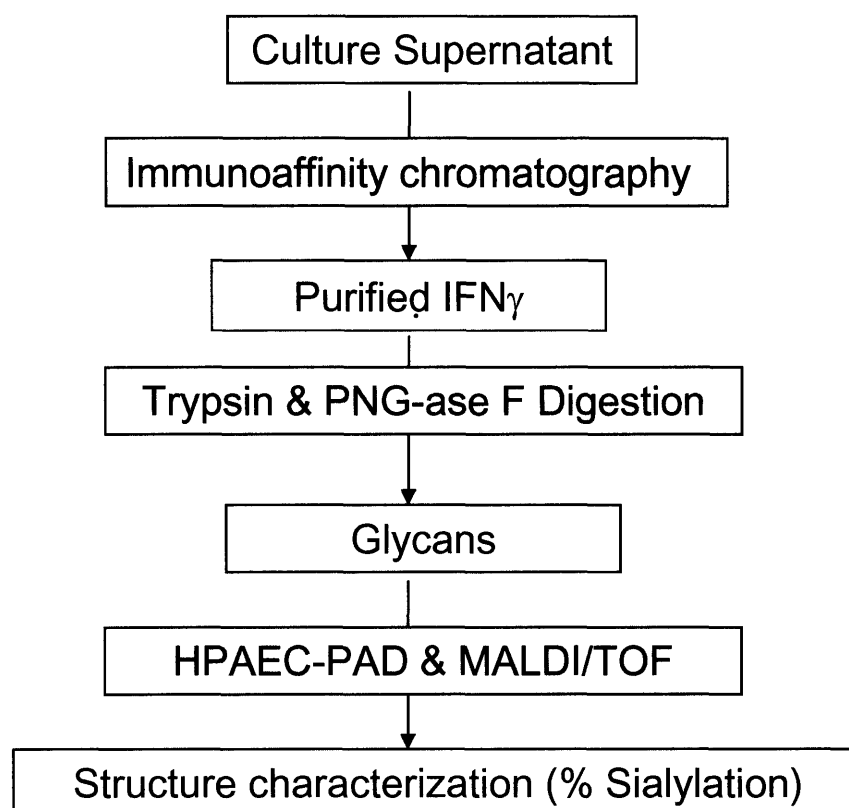


Figure 5.3 Characterization of interferon gamma microheterogeneity.

A more rigorous approach to identify the success of knocking down sialidase was to analyze the structure of the glycoprotein on the sugar level. By analyzing the types of sugars existing at each glycan, percent sialylation can be more accurately quantified. First, IFN γ was purified using immunoaffinity chromatography. Then, IFN γ was trypsinized and treated with Peptide:N-glycosidase F (PNG-ase F) to obtain glycans from Asn-25 and Asn-97 glycosylation sites. The resulting glycans were separated using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Matrix Assisted Laser Desorption /Ionization-Time Of Flight (MALDI-TOF) mass spectrometry was used to reconfirm peaks resolved by HPAEC-PAD.

There are a number of techniques available to select stable cell clones with a high level of plasmid integration. One of the most common approaches is to coexpress a drug resistance marker with the plasmid that contains gene of interest (Hubbard et al., 1994). When the transfected cells uptake and integrate the plasmid, they have a resistance against the drug added in the culture media. On the other hand, those cells that fail to uptake the plasmids will be killed during the drug selection step. There are a few shortcomings with the drug selection technique when sorting stable cell clones. Firstly, the selection process could take a significant amount of time and labor. Secondly, resistance against a drug does not correlate to high expression of plasmid integrated in the genome. As a result, it is hard to distinguish cells with many plasmids from those with just a few. Assaying for the expression of a target gene is another possible selection method. Cells expressing high level of sialidase shRNA should express low levels of sialidase. Assays for target genes are performed at the mRNA or protein levels. With a large number of cells these assays can be time consuming and hard to perform.

Another technique to select stable cell clones with high level of gene expression involves the expression of an anchor protein for modified antibody fragment. A membrane-anchored single chain antibody is coexpressed with the gene of interest. These single chain antibodies then act as hooks to allow for the selection of highly expressing cells with the help of magnetic beads for a rapid selection process (Chesnut et al., 1996). Coexpression of LacZ with the gene of interest is another possible technique to allow rapid selection with LacZ reporter. Despite the simplicity of this approach, additional chemicals in the medium are needed to determine beta-galactosidase activity (Grossman et al., 1997).

To avoid the problems of substrate addition and to expedite the cell selection process, a fluorescence reporter gene can be utilized. Specifically, the coexpression of enhanced green fluorescent protein (eGFP) with gene of interest can assist in determining expression levels. Rapid and straightforward detection of cellular fluorescence with flow cytometer makes this technique invaluable to replace or to complement traditional technique such as drug screening (Vezina et al., 2001; Ozawa et al., 2005). Correlation of the fluorescent signal to the level of silencing is easily accomplished by performing gene-specific assays in addition to fluorescence signal measurement. This correlation then enables sorting of cells with different levels of silencing. Cell sorting via flow cytometry can be performed multiple times to continually select for the cell population of interest. Finally, the use of expressing both the sequence of interest and the eGFP reporter from a single vector avoids the need for cotransfection of two separate plasmids.

In other applications related to CHO cell culture the use of fluorescence reporter has been demonstrated to be a successful high throughput method for stable cell isolation. Ito and coworkers (2000) used GFP techniques to select CHO cells deficient in peroxisome. Yuk et al. (2002) used GFP as a reporter gene for isolating growth-arrested IFN γ producing cells by using a bicistronic vector, encoding both the recombinant protein and GFP reporter gene. Some other examples of using GFP as a reporter gene include determining promoter properties and protein localization studies (Bleve et al., 2005; Green et al., 2005)

In this thesis, we initially combined H1-driven shRNA plasmid with GFP plasmid to produce GFP-containing pol III-driven shRNA plasmid. The plasmid was transfected to CHO cells and

the fluorescence level of sorted cells was correlated to sialidase mRNA knock down. Then GFP-containing shRNA plasmid with a stronger promoter, namely a modified CMV promoter, was constructed and tested. An enzymatic assay using 4MU substrate and real time RT-PCR were used to detect sialidase reduction.

5.3 Results

Stable Silencing of Sialidase using Plasmid-Borne siRNA Expression

After successfully identifying a few efficacious sialidase siRNA sequences, the next step was to integrate these sequences into pol III or pol II driven plasmids. These plasmids, once transfected into the cell, could continuously produce short hairpin RNAs (shRNA). Short hairpin RNAs were further processed into sialidase siRNAs. In our study the pSilencer plasmids utilizing the pol III promoters, U6 and H1 (Ambion), were initially used to drive the expression of sialidase shRNA, because these promoters were found very effective in many cell lines, such as HEK-293, MCF-7, HT-1080, CHO-K1 cells, and many more (Brummelkamp et al., 2002; Malphettes and Fussenegger, 2004; Mori et al., 2004). Transfecting each cell lines with pol III-driven shRNA plasmid was trivial because there were many established methods to introduce plasmid DNA to mammalian cells. Utilizing a positive control such as pEGFP-C1 (BD Biosciences) plasmid would ensure whether transfection efficiency was high or not.

It was found that after transfecting CHO cells with pol III-driven shRNA plasmid, screening for cell lines with reduced sialidase mRNA expression was difficult because drug resistance (resulting from the hygromycin resistance sequence in the plasmid) did not guarantee high copies of plasmid integrated into each CHO cell. Nearly 1000 clones were tested, and we were unable to obtain cell lines with over 50% sialidase activity reduction using these U6 and H1 promoter-incorporating plasmids. As shown in Table 5.1 and Table 5.2, three clones derived from U6 promoter-driven shRNA plasmid (Clone B19, B21, A251) and three clones derived from H1 promoter-driven shRNA plasmid (Clone A78, B91, A191) were found to have about 18-23% sialidase activity reduction.

Table 5.1 Sialidase activity of various attached single cell colonies resulted from transfection of sialidase shRNA plasmid utilizing U6 promoter.

1×10^5 CHO-IFN- γ cells were transfected with U6 promoter-driven shRNA plasmid. Clone A, B, C utilized sialidase siRNA sequence S1 while clone D, F utilized sialidase siRNA sequence S5. After selection with hygromycin, single cell colonies were picked and expanded to reach over 1×10^6 cells. Sialidase activity was performed on cell lysate utilizing 4MU-NeuAc fluorescence substrate. Three independent measurements were performed and normalized against fluorescence of untransfected parent cell lines. For normalization purposes, sialidase activity from parent cell line was set to be 1. Over 500 clones were selected and ten of them were displayed as representative. Clone B19, B12, A251 were taken for further analysis.

Clone	Normalized sialidase activity
U6-B19	0.76 ± 0.07
U6-B12	0.76 ± 0.02
U6-A251	0.82 ± 0.01
U6-F171	0.88 ± 0.01
U6-F128	0.89 ± 0.02
U6-C15	0.92 ± 0.02
U6-A127	0.94 ± 0.02
U6-A1	0.96 ± 0.01
U6-D17	0.99 ± 0.02
U6-C13	0.99 ± 0.02
Parent	1.00

Table 5.2 Sialidase activity of various attached single cell colonies resulted from transfection of sialidase shRNA plasmid utilizing H1 promoter.

1×10^5 CHO-IFN- γ cells were transfected with H1 promoter-driven shRNA plasmid. Clone A, B, C utilized sialidase siRNA sequence S1 while clone D, E, F utilized sialidase siRNA sequence S5. After selection with hygromycin, single cell colonies were picked and expanded to reach over 1×10^6 cells. Sialidase activity was performed on cell lysate utilizing 4MU-NeuAc fluorescence substrate. Three independent measurements were performed and normalized against fluorescence of untransfected parent cell lines. For normalization purposes, sialidase activity from parent cell line was set to be 1. Over 500 clones were selected and ten of them were displayed as representative. Clone A78, B91, A191 were taken for further analysis.

Clone	Normalized sialidase activity
H1-A78	0.72 ± 0.01
H1-B91	0.76 ± 0.03
H1-A191	0.77 ± 0.02
H1-B7	0.78 ± 0.03
H1-B181	0.88 ± 0.02
H1-C1	0.91 ± 0.06
H1-C18	0.91 ± 0.02
H1-E15	0.92 ± 0.03
H1-F32	0.94 ± 0.05
H1-D9	0.98 ± 0.07
Parent	1.00

Before proceeding to quantitate sialic acid content with laborious analytical technique, sustainability of sialidase reduction must be proved utilizing a fluorescent artificial substrate. Unfortunately, the sialidase knockdown was not maintained after the cells were passaged numerous times, implying RNA pol III promoters are not strong enough to generate hairpin RNA continuously in CHO cells. The sialidase activity of clone A191 and A251 immediately returned to parent CHO-IFN- γ levels after one passages (data not shown). Clone B19, B12, A78, and B91 showed variable sialidase suppression up to 6 generations, ranging from 2-28% sialidase reduction. None of these pol III-driven shRNA plasmid transfected clones established significant sialidase reduction beyond 10 generations (Figure 5.4). These cell lines were not suitable for further analysis because stability was a necessity for translation to industrial settings.

In order to successfully knock down sialidase activity with the RNAi method, it was important to have sufficient sialidase siRNA available intracellularly to inhibit translation of sialidase mRNA. It has been demonstrated previously by Ui-Tei and coworkers that CHO-K1 cell lines required high dose of chemically synthesized siRNA to knock down *P. pyralis luc* mRNA levels (Ui-Tei et al., 2000). Compared to *Drosophila* S2 cells, CHO-K1 cells required 2500 times more *P. pyralis luc* siRNA. This phenomenon suggested the requirement of high level of siRNA in order to make RNAi a viable gene knock down technique for CHO cells. To constitutively produce siRNA *in vivo*, short hairpin RNA (shRNA) produced by plasmids were generally used to generate siRNA, and these plasmids were typically driven by pol III promoters, such as U6 and H1 promoters.

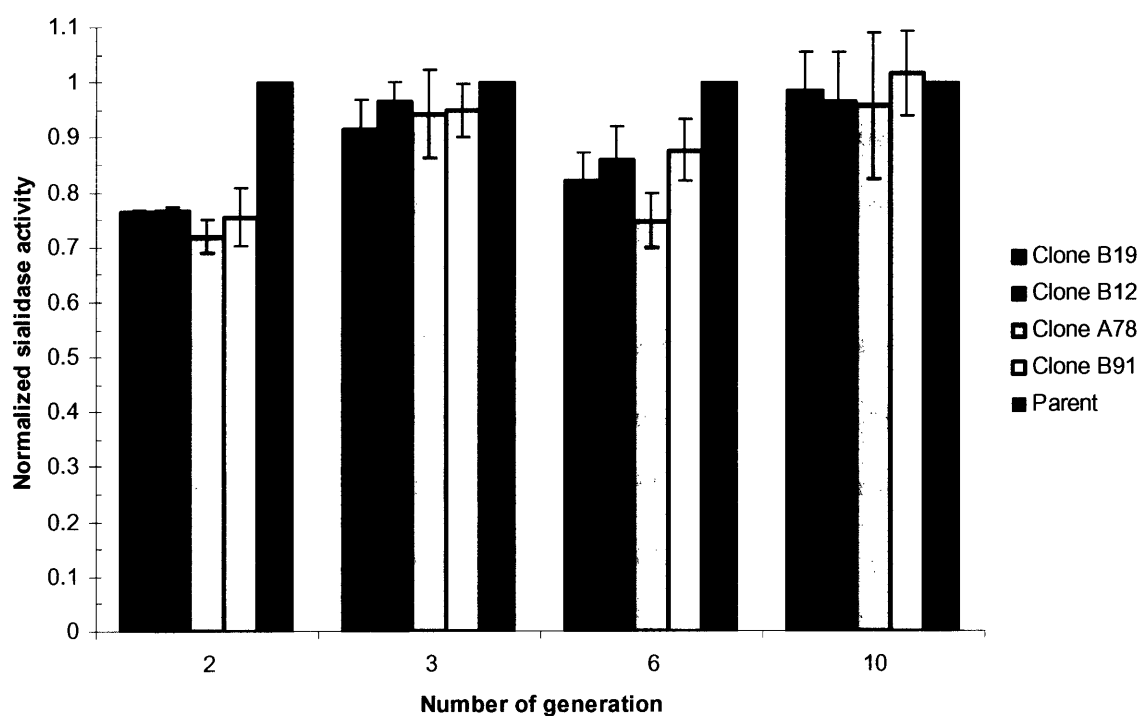


Figure 5.4 Stability analysis of sialidase suppression on attached single cell clones transfected with shRNA plasmid utilizing U6 and H1 promoter

Four of single cell clones exhibiting reduced sialidase activity from shRNA plasmid transfection were expanded and their sialidase activity was measured throughout numerous passages. Clone B19 and B12 contained U6 promoter while clone A78 and B91 contained H1 promoter. 1×10^6 cells were collected and lysed for each generation. For normalization purposes, sialidase activity from parent cell line was set to be 1.

Based on our success in utilizing chemically synthesized siRNA to transiently knock down sialidase activity, we believed the failure to identify stable clones was attributed to the low transcription of siRNAs generated by the assistance of pol III promoters. A recent work by Mori and Satoh in Japan confirmed our hypothesis that pol III promoter was not a strong promoter for CHO cell lines (Mori et al., 2004). U6 promoter was employed by Mori to generate siRNA to knock down Fut8 mRNA level and after a few rounds of drug screening, no stable puromycin-resistant cell clones with significant reduction in Fut8 mRNA level was established. The random nature of plasmid integration into the chromosome could also contribute to the difficulties in finding stable cell clones with reduced sialidase expression level. It was well established that gene integration into different areas of chromatin yielded different expression of the inserted gene (Wilson et al., 1990; Barnes et al., 2003). If the plasmid was integrated in highly condensed and transcriptionally inactive areas, such as heterochromatin area, high plasmid copy numbers were needed to retain shRNA expression from the pol-III driven plasmid. A plasmid transfection targeted into chromosome areas with high expression levels could potentially compensate the weak nature of pol III promoter in CHO cells. Alternatively, a stronger promoter could be utilized.

A pol II, modified CMV promoter was subsequently used to drive the long-term expression of sialidase siRNA. It is known that the CMV promoter is stronger than the other promoters and modification of the CMV promoter was recently shown to drive high levels of functional siRNAs (Foecking and Hofstetter, 1986; Xia et al., 2002). Initially, out of 300 clones generated from this modified CMV plasmid (Ambion), four clones were found to reduce sialidase activity by 2-3 folds (Figure 5.5).

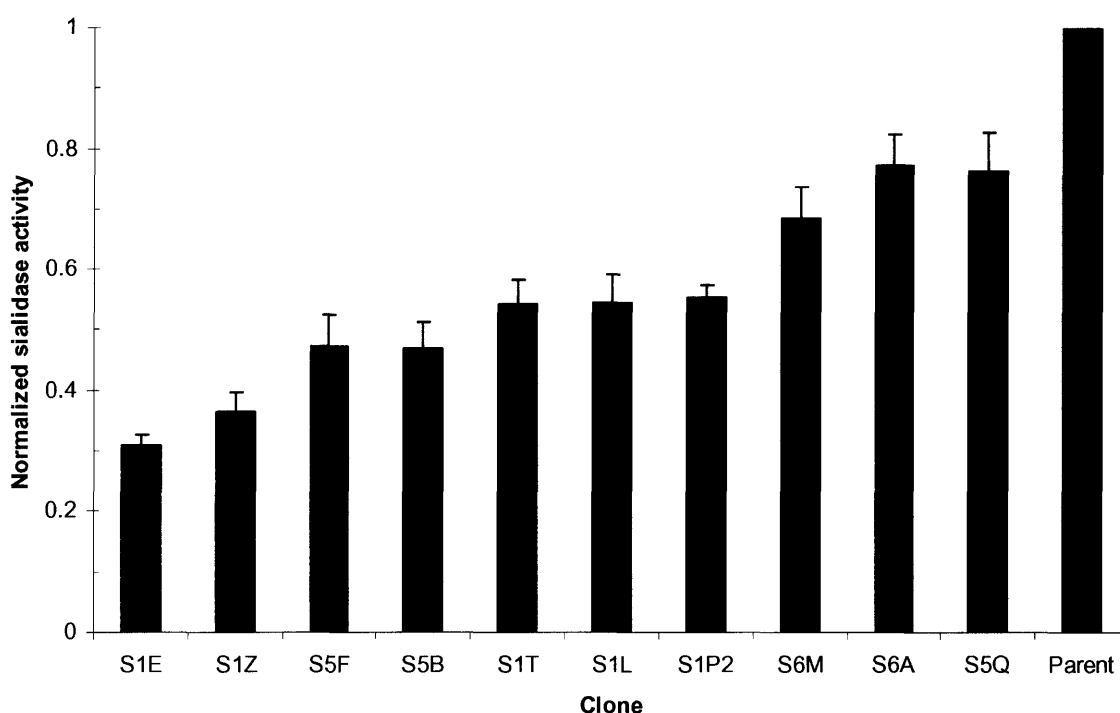


Figure 5.5 Sialidase activity of various attached single cell colonies resulted from transfection of sialidase shRNA plasmid utilizing modified CMV promoter.

1×10^5 CHO-IFN- γ cells were transfected with modified CMV promoter-driven shRNA plasmid. Clone S1 utilized sialidase siRNA sequence S1 while clone S5 and S6 utilized sequence S5 and S6 respectively. After selection with hygromycin, single cell colonies were picked and expanded to reach over 1×10^6 cells. Sialidase activity was performed on cell lysate utilizing 4MU-NeuAc fluorescence substrate. Three independent measurements were performed and normalized against fluorescence of untransfected parent cell lines. For normalization purposes, sialidase activity from parent cell line was set to be 1. Over 250 clones were selected and ten of them were displayed as representative. Clone S1E, S5F and S5B were expanded for further analysis because clone S1Z were contaminated.

We found that CHO cells transfected with shRNA plasmid driven by pol II promoters (modified CMV promoters) generally exhibited better sialidase knock down as compared to CHO cells transfected with shRNA plasmid driven by pol III promoters. In fact, there were 25 clones with over 50% sialidase activity reduction on these CMV promoter-driven cell lines (data not shown). The best three clones (clone S1E, S5B, and S5F) were further analyzed by growing them for longer periods of time in batch mode. Clone S1Z was not carried forward due to contamination by bacterial. Clone S1E (which has plasmid encoding siRNA sequence S1) and clone S5F (which has plasmid encoding sequence S5) were found to maintain low sialidase activity as compared to the parent cell throughout the cell culture even when the cells began to die (Figure 5.6). This was observed consistently from the lag phase, growth phase, stationary phase, and death phase of the cell culture. On the other hand, clone S5B (which has the plasmid encoding sequence S5) did not have a consistent reduction in sialidase activity level. After 24 hours (during growth phase), clone S5B slowly exhibited sialidase activity close to the parent cell line and after 72 hours (during stationary phase), it exhibited sialidase activity close to the clone S1E and S5F. During the death phase of cell culture, clone S5B's sialidase activity increased to the level close to the parent cell line. Due to its inconsistency of sialidase activity, we did not carry clone S5B for further analysis.

Stability Analysis of Stably Transfected Cell

It is essential to ensure that sialidase knock down continued from generation to generation. Cell lines in which sialidase activity reverted to the activity of untransfected CHO cells would not be a useful because increased sialidase activity would correspond to lower sialic acid content. In

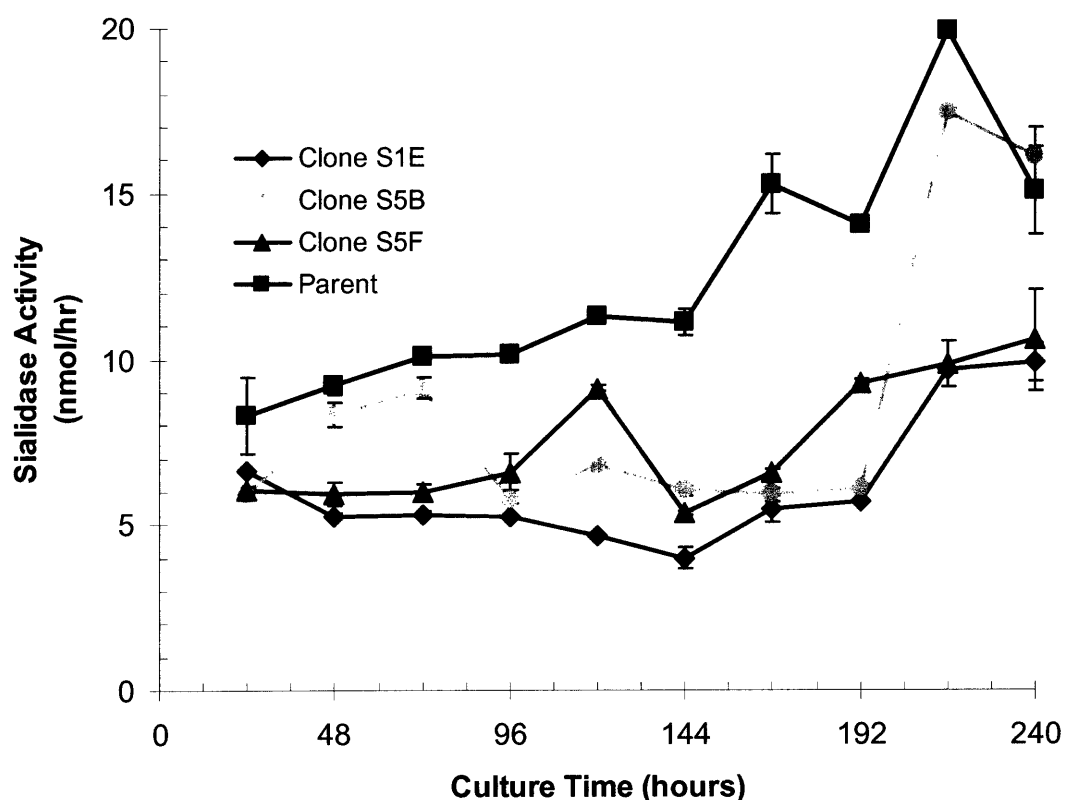


Figure 5.6 Sialidase activity profile for three attached single cell clones transfected with shRNA plasmid containing modified CMV promoter

Sequence S1 and S5 were incorporated into separate plasmids used to create stable cell lines that continuously produced sialidase siRNA. Clones S1E (which has plasmid encoding siRNA sequence S1) and S5F (which has plasmid encoding sequence S5) were found to maintain low sialidase activity throughout various phases of batch cell culture. On the other hand, clone S5B showed inconsistent sialidase reduction during the growth phase and the death phase of cell culture, it exhibited sialidase activity similar to that of the parent cell.

this stability study, S1E cell lines and parent cell lines were analyzed during their log growth phase from generation to generation. It was found that the reduction in sialidase activity possessed by S1E cell lines continued to exist after 25 generations (Figure 5.7). On average, the sialidase activity on S1E cell lines were lower by 2 – 2.5 folds. The sialidase reduction over 25 generations was not due to clonal variability because during our studies, a parallel sialidase activity assay experiment on CHO-IFN γ cell lines was not successful in identifying cell lines with significant (>50%) sialidase activity reduction.

To ensure that the sialidase knock down was due to siRNA expressed by pol II-driven shRNA plasmids and to demonstrate that these plasmids were integrated into the genome, PCR was performed on genomic DNA collected from clone S1E and parent cell lines. The primers were designed to amplify a region of the plasmid that contained a part of CMV promoter sequences and a part of sialidase hairpin oligonucleotide. If integration event occurred, the PCR reaction using clone S1E genomic DNA as a template should yield a PCR product with expected base pair length. As shown in Figure 5.8, PCR reaction on genomic DNA originated from parent cell did not yield DNA at the expected base pair lengths. On the other hand, genomic DNA isolated from both attached S1E clone and S1E clone that has been adapted to serum-free suspension cell lines successfully produced cDNA of CMV promoter and hairpin oligonucleotide after cycles of PCR reaction. This indicated that integration of the pol II-driven shRNA plasmid into CHO genome occurred and the stability of the clone S1E cell lines to suppress sialidase activity originated from siRNA generated by this integrated plasmid.

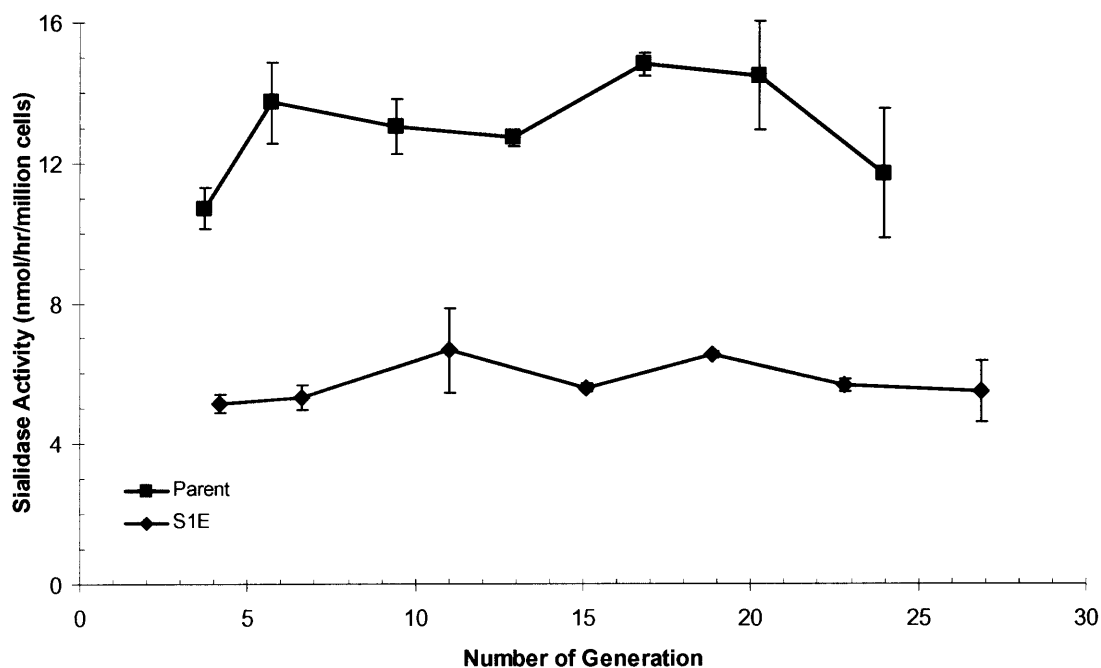


Figure 5.7 Stability analysis of sialidase suppression on attached single cell clones transfected with shRNA plasmid utilizing modified CMV promoter: Fluorescence assay

It is essential to ensure sialidase knock down was still occurring generation after generation. Cell lines which sialidase activity restored to the activity of untransfected CHO cells would not be a useful cell line because an increased in sialidase activity would correspond to a lower sialic acid content. In this stability study, S1E cell lines and parent cell lines were analyzed during their growth phase one generation after another generation. It was found that the reduction in sialidase activity possessed by S1E cell lines still existed over 25 generations, indicating the stability in sialidase suppression

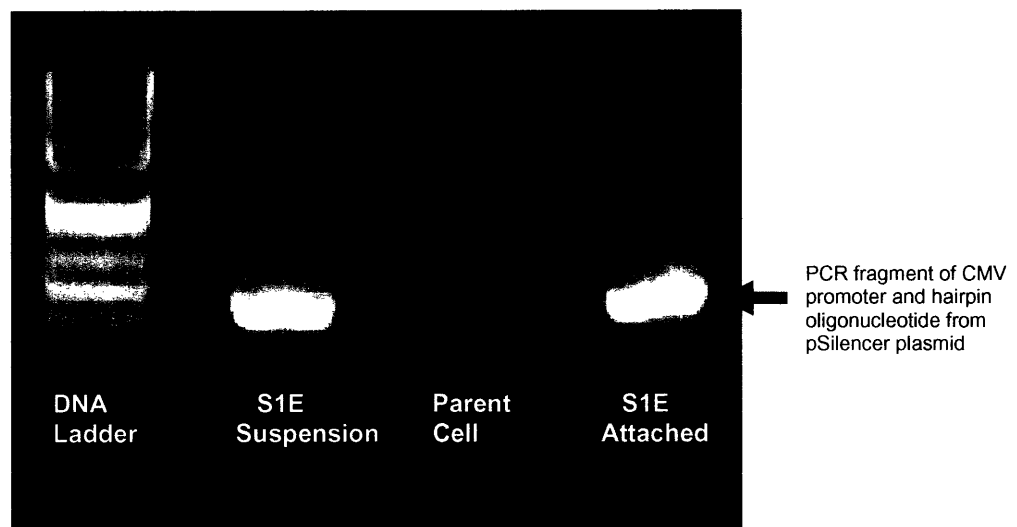


Figure 5.8 Stability analysis of sialidase suppression on single cell clones transfected with shRNA plasmid utilizing modified CMV promoter: PCR assay

1×10^7 cells were harvested and their genomic DNAs were isolated using Wizard SV genomic DNA purification kit (Promega). These genomic DNAs were used as a template for a PCR reaction which primers were designed based on a fragment of pSilencer plasmid. This fragment consisted of CMV promoter region and hairpin oligonucleotide region of annealed pSilencer plasmid. Genomic DNA from parent cell should not get amplified.

Viability and Glycoprotein Titer of Stably Transfected Cell

siRNAs were used to decrease sialidase activity partially because different levels of silencing can be obtained by selecting different siRNA sequences. Gene knock out was avoided at first because the sialidase gene might be important to cell viability. A previous work by Sato and Miyagi (1996) and more recently by Fanzani and coworkers (2003) indicated that cytosolic sialidase could be important in myoblast differentiation by desialylating glycoconjugates. In mouse B16 melanoma cell lines, cytosolic sialidase level was linked to metastasis and cell motility (Sawada et al., 2003). Since cytosolic sialidase level of CHO cells has not been characterized extensively, completely knocking out cytosolic sialidase might not be the best idea and any successful silencing must be followed by cell viability analysis to ensure normal cell growth.

In this study, clones S1E, S5B, and S5F were compared with parent cell lines during 2-weeks of batch culture. Both clones exhibited similar growth profiles as shown by similar lengths of lag phases, exponential growth phases, and death phases (Figure 5.9). It was observed that clone S1E exhibited a maximal cell density 40% greater than the parent cell line while clone S1B exhibited a maximal cell density 40% lower than parent cell line. Specific growth rate was estimated based on the growth curve data during exponential growth phase. As shown in Table 5.3, S1E cell lines exhibited a specific growth rate 10% higher than parent cells while S5B and S5F cell lines exhibited specific growth rate 13% and 15% lower than parent cells respectively. These values were acceptable within the specific growth rate estimated from typical batch culture performed in our lab (Fox, 2005). The lower specific growth rate on clone S5B and S5F were most likely due to the toxicity of hygromycin supplemented within the cell culture media rather than gene

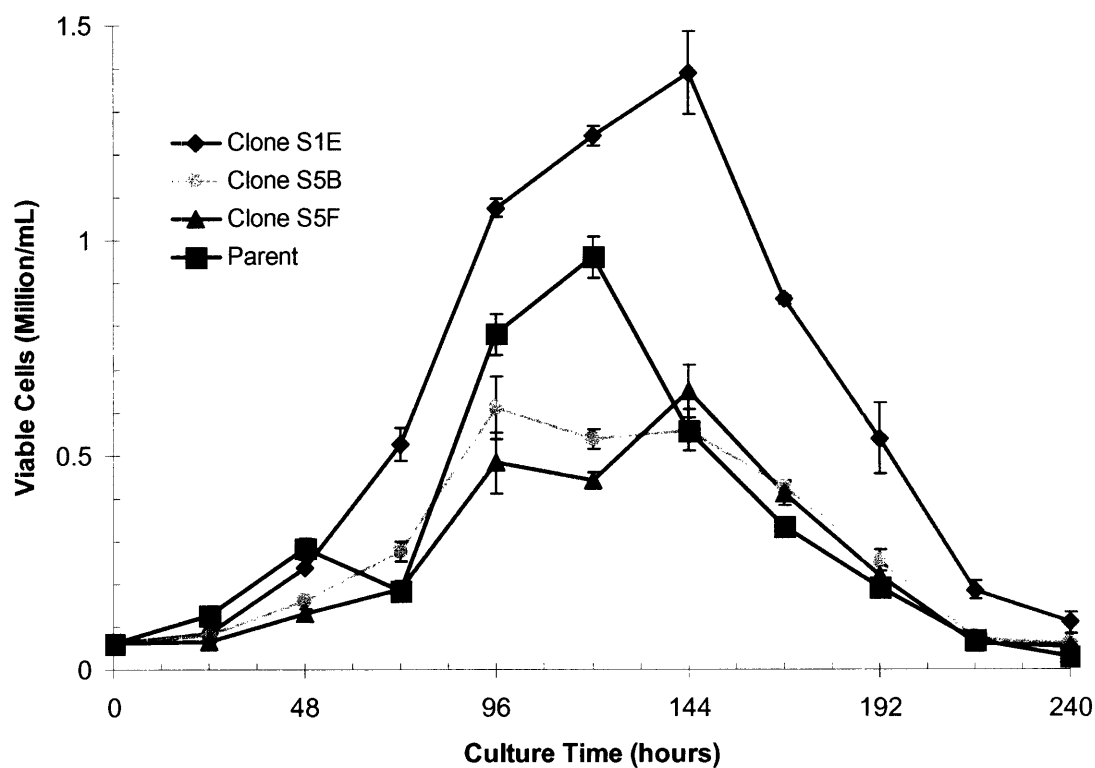


Figure 5.9 Viable cell densities of attached single cell clones transfected with shRNA plasmid utilizing modified CMV promoter

One of the main concerns regarding gene knock down is the potential detrimental effect towards cell viability. In this experiment, cell enumeration was done throughout cell culture over numerous passages. It was observed that cells transfected with siRNA-producing plasmid exhibited similar grow profile as shown by similar length of lag phase, growth phase, and death phase.

Table 5.3 Average specific growth rates for batch culture of attached single cell clones transfected with shRNA plasmid compared with parent cells.

Specific growth rate for each cell clone was determined from three different batch cultures. Estimate was performed from viable cell numbers enumerations obtained during exponential growth phase.

Cell Lines	Specific Growth Rate (hr ⁻¹)
S1E	0.0354 ± 0.0071
S5B	0.0281 ± 0.0055
S5F	0.0274 ± 0.0123
Parent	0.0322 ± 0.0014

knock down. At this initial stage of the study, hygromycin supplementation was still necessary to ensure only cell lines transfected with pol II-driven shRNA plasmid persisted throughout generations. Upon removal of hygromycin after numerous passaging, clone S5F specific growth rate was indistinguishable from the specific growth rate of parent cell lines (data now shown). Based on this data, it was concluded that knocking down sialidase activity does not have a detrimental effect on cell viability.

Another important issue to consider is the titer of the glycoprotein (IFN γ) produced from the CHO cells. Plasmid integration occurs randomly within the genome and although the probability of integration of pol II-driven shRNA plasmid in the IFN γ site was very low, it is important to ensure that the cell continues to produce glycoprotein at acceptable levels. ELISA (Biosource International Inc) was used to quantify IFN γ titer during cell culture and average specific productivity was estimated from the slope of cumulative IFN γ concentration plot versus integrated viable cell area. Parent cell line's specific productivity of IFN γ was estimated to be 0.81 pg/cell/day while the specific productivity of clone S1E, S5B, S5F were estimated to be 0.65, 0.63 and 0.95 pg/cell/day respectively (Figure 5.10). Although each reduced sialidase cell line had different specific productivity, this variation was still within the range of specific productivity of the CHO-IFN- γ utilized by other researchers in the lab (Nyberg, 1998; Fox, 2005). In addition, methotrexate, which was used to amplify IFN γ expression, was known to increase the frequency of chromosome arrangements arising from translocations and homologous recombination (Yoshikawa et al., 2000). As a result, CHO cells subjected to methotrexate for gene amplifications possessed unstable karyotype and specific productivity from batch to batch could vary even for cell populations derived from a single clone.

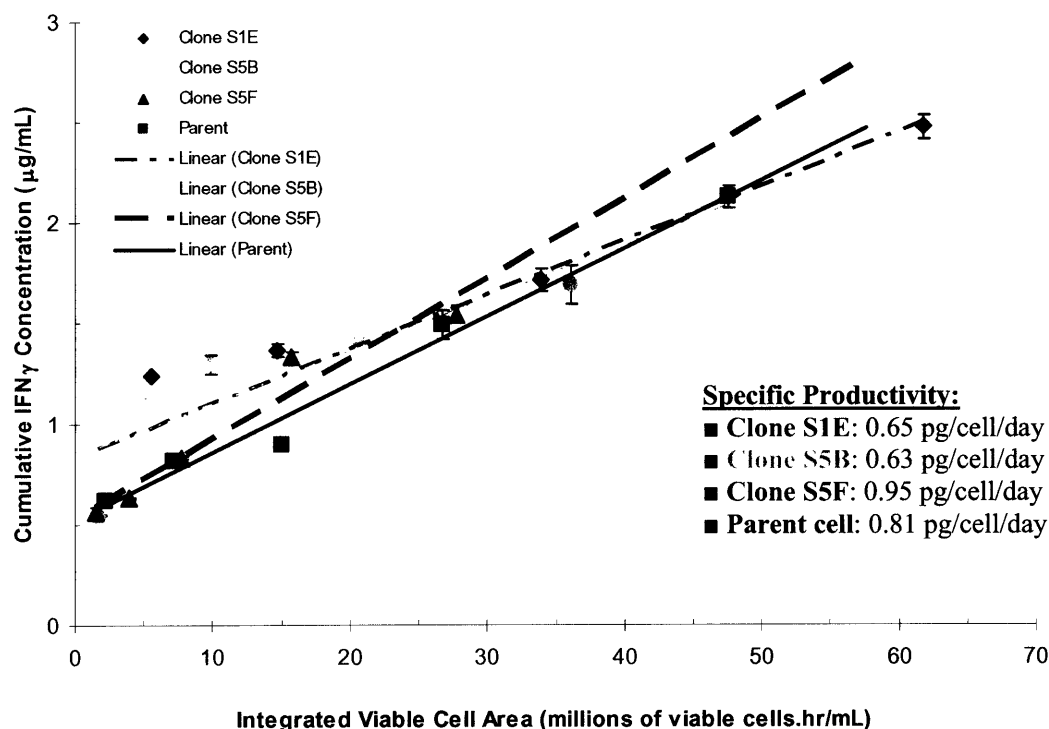


Figure 5.10 Glycoprotein (IFN γ) productivity of attached single cell clones transfected with shRNA plasmid utilizing modified CMV promoter during batch culture of CHO-IFN- γ

By knocking down sialidase, it is important to ensure that the plasmid integration into the chromosome does not affect the glycoprotein expression by CHO cells. The amount of IFN γ was quantified throughout cell culture by using ELISA technique and plotted against integrated viable cell area. Regression analysis was performed on the data points to estimate specific productivity for each cell line. It was found that stably transfected cells still produced IFN γ in the amount comparable to parent cell.

Sialic Acid Content Analysis and Macroheterogeneity Analysis of IFN γ

The effectiveness of RNAi technique to knock down sialidase has been demonstrated via sialidase transcript and protein activity measurement. However, the more important issue still remains: do lower levels of sialidase activity result in improvement of sialic acid content of the glycoprotein throughout cell culture? Of particular interest, is the sialic acid content of IFN γ maintained during the death period when cytosolic sialidase is released into the culture medium (Gramer et al., 1995; Munzert et al., 1996; Gu et al., 1997; Gramer, 2000). Theoretically, silencing cytosolic sialidase would result in constant sialic acid content profile throughout cell culture similar to the benefit of adding 2,3-D inhibitor.

In the experiment presented below, IFN γ was collected and purified from two distinct phases: growth phase, when the cells were still actively growing and death phase, when the nutrient depletion occurred and the cells began to die. As shown in Figure 5.11, at 96 hours (which is still within the growth phase of the cells), parent cells maintained high sialic acid content per IFN γ molecule, 3.32 mole sialic acid/mole IFN γ . However, as the cells began to die, sialic acid content decreased significantly at a rate of 0.05 moles of sialic acid/moles of IFN γ / day. This phenomenon has also been previously observed by many researchers (Gramer et al., 1995; Gu et al., 1997). This resulted from the release of cytosolic sialidase into the culture supernatant which in turn cleave the sialic acid from IFN γ .

On the other hand, this phenomenon did not occur with clone S1E and S5F. In fact, sialic acid remained at 3.04 ± 0.09 mole sialic acid/mole IFN γ for clone S1E and 3.14 ± 0.19 mole sialic acid/mole IFN γ for clone S5F during growth phase and death phase, thereby demonstrating the

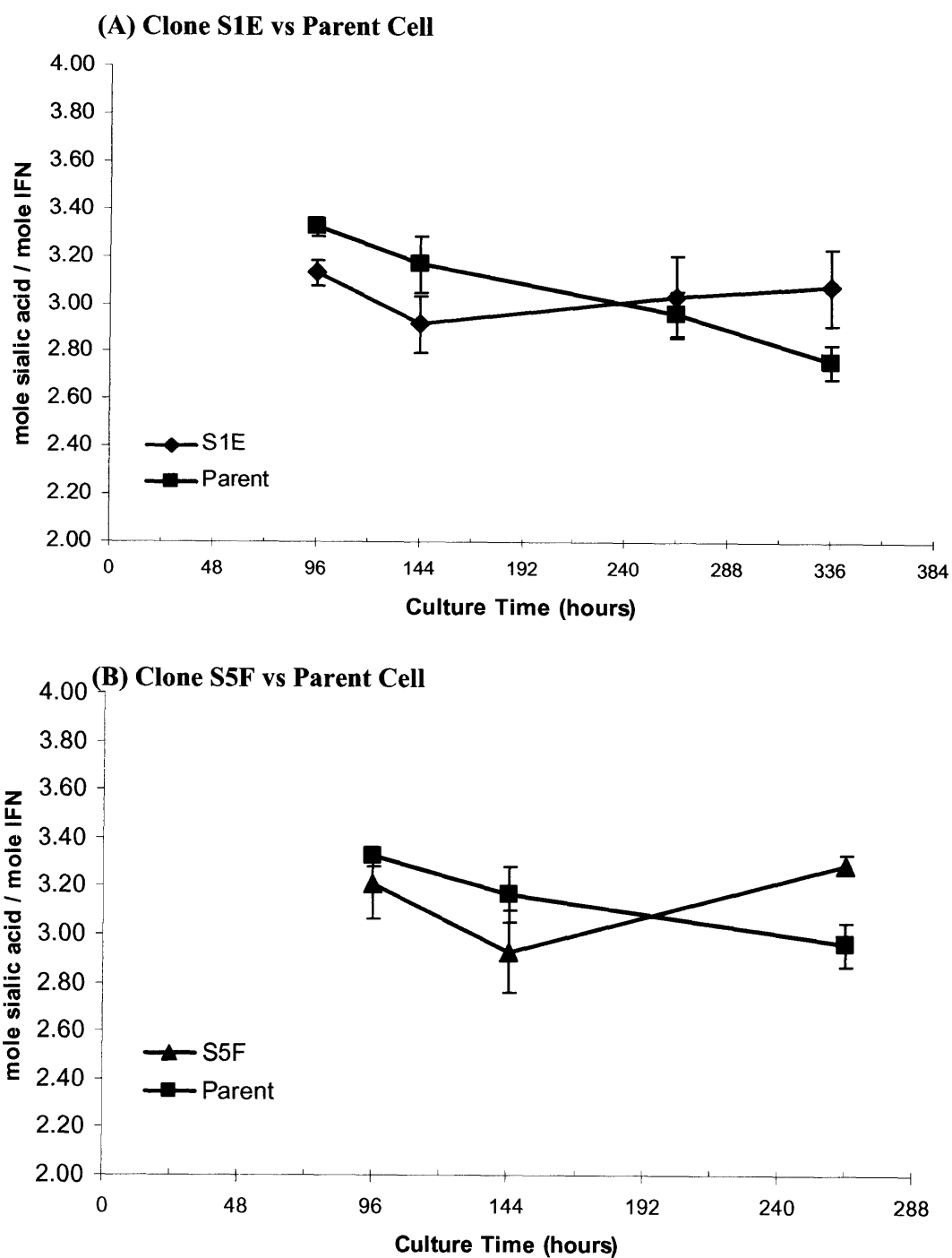


Figure 5.11 Sialic acid content of attached single cell clones transfected with shRNA plasmid utilizing modified CMV promoter

In parent cell lines, as culture time increases, CHO cells began to die and sialidase was released in the supernatant. As a result, the amount of sialic acid per glycoprotein decreased as a function of increasing culture time. On the other hand, for S1E and S5F cell line, although the number of viable CHO cells gradually decreased, the number of sialic acid remained relatively the same as the number of sialic acid early in the cell culture.

effectiveness of siRNA in knocking down sialidase activity and maintaining sialic acid content throughout the cell cultures. This result is comparable with the relatively constant sialic acid profile obtained when the sialidase inhibitor (2,3-dehydro-2-deoxy-N-acetylneuraminic acid) was added to the cell supernatant (Gu et al., 1997).

During this study, we observed that although sialidase activity was not completely eliminated (Figure 5.6), the relatively constant sialic acid content was maintained throughout cell cultivation times (Figure 5.11). Except during the onset of the cell death, the effect of sialidase siRNA on sialic acid content attached to the end of glycoprotein's glycan was very similar to the inhibition profile by the chemical inhibitor. We believe that the fluorescence substrate that we utilized to monitor cytosolic sialidase knock down (4MU-NeuAc) could be cleaved by other types of sialidase. It was demonstrated by the Japanese researchers that 4MU-NeuAc was cleaved by either lysosomal sialidase, membrane-associated sialidase, or cytosolic sialidase isolated from rat (Miyagi and Tsuki, 1984; Miyagi et al., 1993; Hasegawa et al., 2001). Considering the close sequence homology between rat and hamster, we believe that other types of sialidase exist in CHO. As a result, measuring sialidase originated from CHO lysate was the same as measuring total sialidase activity originating from lysosomes, membranes, and cytosols. A knock down of 2-3x in sialidase activity measured from lysate corresponded to 2-fold or more knock down in cytosolic sialidase activity. In addition to performing sialidase activity assay, real-time quantitative PCR assay was also performed to quantify the extent of sialidase mRNA degradation. As shown by Figure 5.12, cytosolic sialidase mRNA of clone S1E was consistently 4-5 folds lower than the cytosolic sialidase mRNA of parent cells. Due to the

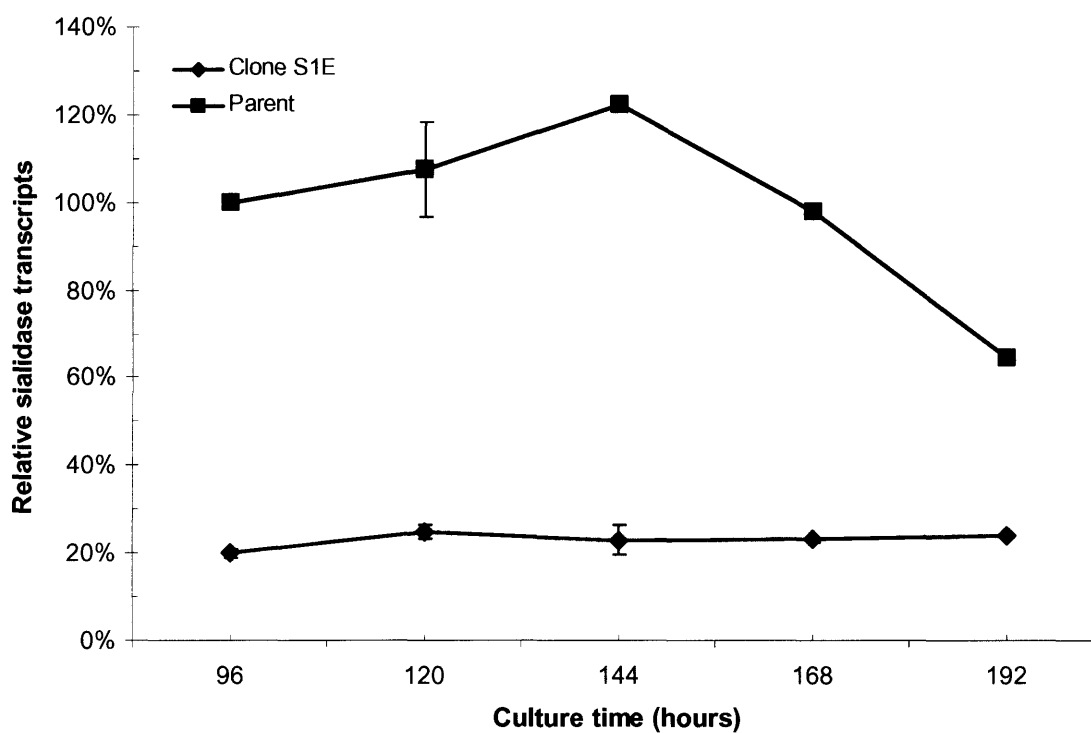


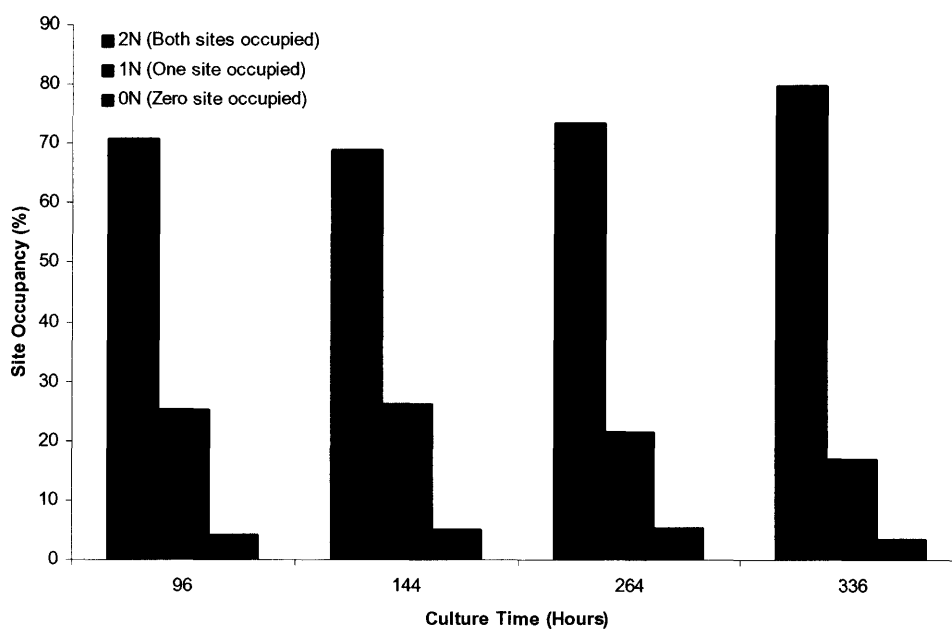
Figure 5.12 mRNA quantification of stable attached single cell clones transfected with shRNA plasmid utilizing modified CMV promoter

Total RNA was isolated from clone S1E IFN- γ cells at various different culture time and two step RT-PCR was performed. β -actin mRNA was utilized as internal control and reported sialidase mRNA level from quantitative PCR assay was normalized with β -actin mRNA level.

indiscriminant nature of fluorescence substrate to any types of sialidase, the more specific way to monitor cytosolic sialidase knock down was to quantify the extend of cytosolic sialidase mRNA degradation by designing primers for real-time quantitative PCR very carefully. Utilizing RNAi technique, mRNA was cleaved into at least two fragments. PCR primers were designed to flank the target sequence (which is the portion of the sialidase sequence used to design shRNA oligonucleotide) and the sequence following the target sequence (but not included in the design of shRNA). That way, PCR primers would correctly produce no amplification product when siRNA successfully attacked target sequence and cleaved the mRNA into at least two regions. On the other hand, if PCR primers only attempted to amplify a region in which cleavage did not occur, PCR would falsely result in high amplification that would not represent the real potency of RNAi technique.

Another important set of studies was performed to explore the distribution of glycoforms throughout the cell culture. Although sialidase activity relates solely to the cleavage of terminal sialic acid capping on the glycans of IFN γ , it is crucial to ensure that reduction of sialidase activity does not cause significant macroheterogeneity change in IFN γ . Theoretically, macroheterogeneity was modulated by other factors that did not include glycosidase such as: oligosaccharyltransferase activity and oligosaccharyl dolichol availability (Shelikoff et al., 1996). As demonstrated by Figure 5.13, glycan site occupancy of IFN γ remained relatively unchanged when sialidase mRNA was reduced. This phenomenon was also observed throughout the various phases of cell culture (i.e. growth phase and death phase). This study confirmed that glycan site distribution was not modulated by cytosolic sialidase and therefore sialidase knock down indeed did not alter the glycan site distribution of IFN γ .

(A) Clone S1E



(B) Parent Cell

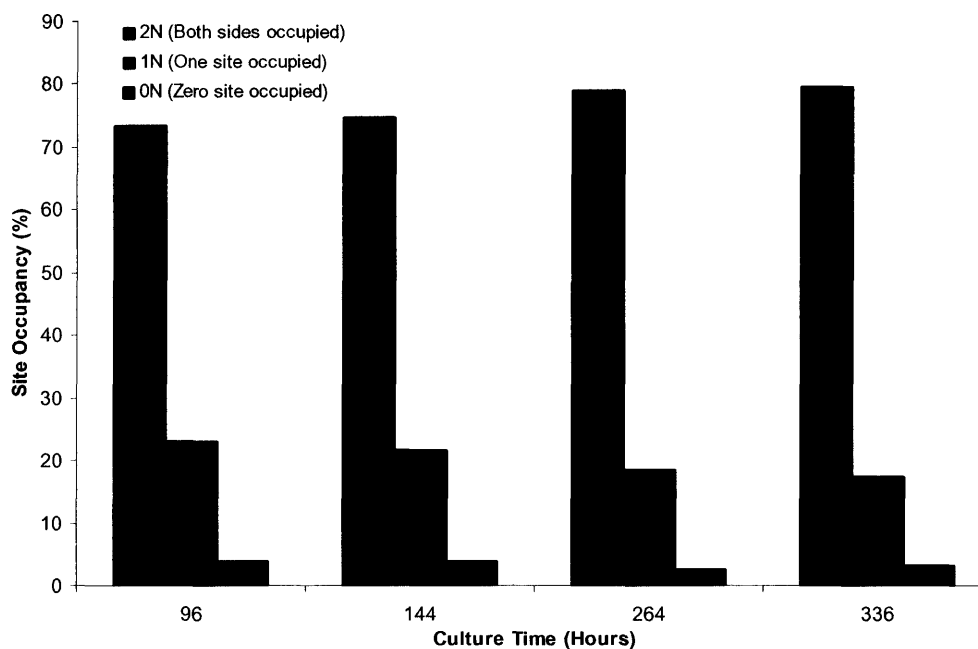


Figure 5.13. Glycan site occupancy of IFN γ from stably transfected clone and parent Cell



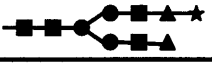




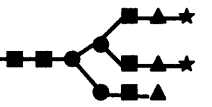
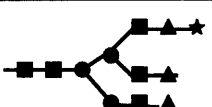


There are two potential sites for N-linked glycosylation in IFN γ , Asn²⁵ and Asn⁹⁷. Therefore, IFN γ can be either occupied in both sites (2N), one site (1N), or unoccupied (0N). In theory, sialidase did not modulate glycan site distribution. With MEKC study, we demonstrated there were indeed no significant differences in site occupancy of Clone S1E and Parent Cell throughout cell cultures.

Microheterogeneity Analysis of IFN γ

To gain more insights on the effect of sialidase knock down to the glycoforms of IFN γ , microheterogeneity analysis using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and MALDI/TOF mass spectrometry (MS) was performed. HPAEC-PAD is a very useful technique to resolve different glycoform structures based on the retention time of heterogeneously sialylated glycoprotein (Rohrer et al., 1998; Rohrer, 2000). Sialic acid is highly negatively charged sugar. As a result, a highly sialylated glycoprotein has longer retention time due to stronger interaction with the positively charged stationary phase (Rohrer, 2000). Chromatograms of HPAEC-PAD analysis are analyzed by matching the retention time of each peak with standards. When standards are not available, MALDI/TOF MS is used to identify the chemical structure of the glycoforms.

Table 5.4 showed the microheterogeneity structures of IFN γ as identified by HPAEC-PAD and MALDI/TOF MS analysis. Chromatograms of resolved glycans obtained from HPAEC-PAD were shown in Figure 5.14 and Figure 5.15. MALDI/TOF MS was able to identify more glycoforms as compared to HPAEC-PAD because success in HPAEC-PAD analysis strongly relied on the availability of standards. Nevertheless, MALDI/TOF resolved peaks cannot be used to estimate sialylation percentage of glycoproteins accurately because the negatively charged and labile terminal sialic acids are often lost during highly energetic MALDI process (Mechref and Novotny, 1998; Huang and Riggan, 2000). One solution to avoid sialic acid instability issues during MALDI process was to remove sialic acid residue using sialidase (Mechref and Novotny, 1998). However, this approach is not useful for our analysis because quantification of sialylation

Table 5.4 Microheterogeneity structures of interferon gamma glycans observed (+) utilizing HPAEC-PAD (AEC) and MALDI-TOF analysis of permethylated glycans (MS).

Glycan structure	Parent CHO-IFN		Clone S1E		Parent CHO-IFN		Clone S1E	
	Growth Phase		Growth Phase		Dead Phase		Dead Phase	
	HPAEC	MS	HPAEC	MS	HPAEC	MS	HPAEC	MS
High mannose								
	+	+	+	+	+	+		+
Complex biantennary ^a								
	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
		+						
	+	+	+	+	+	+	+	+
								
Complex triantennary ^a								
	+	+		+	+	+	+	+
		+		+		+		+
				+		+		+
Complex tetraantennary ^a								
	+						+	+
						+		+

^a Non-fucosylated structures are depicted in this figures although fucosylated structures were also detected in each scenarios. (■) represents N-acetylglucosamine, (●) represents mannose, (▲) represents galactose, and (◆) represents sialic acid.

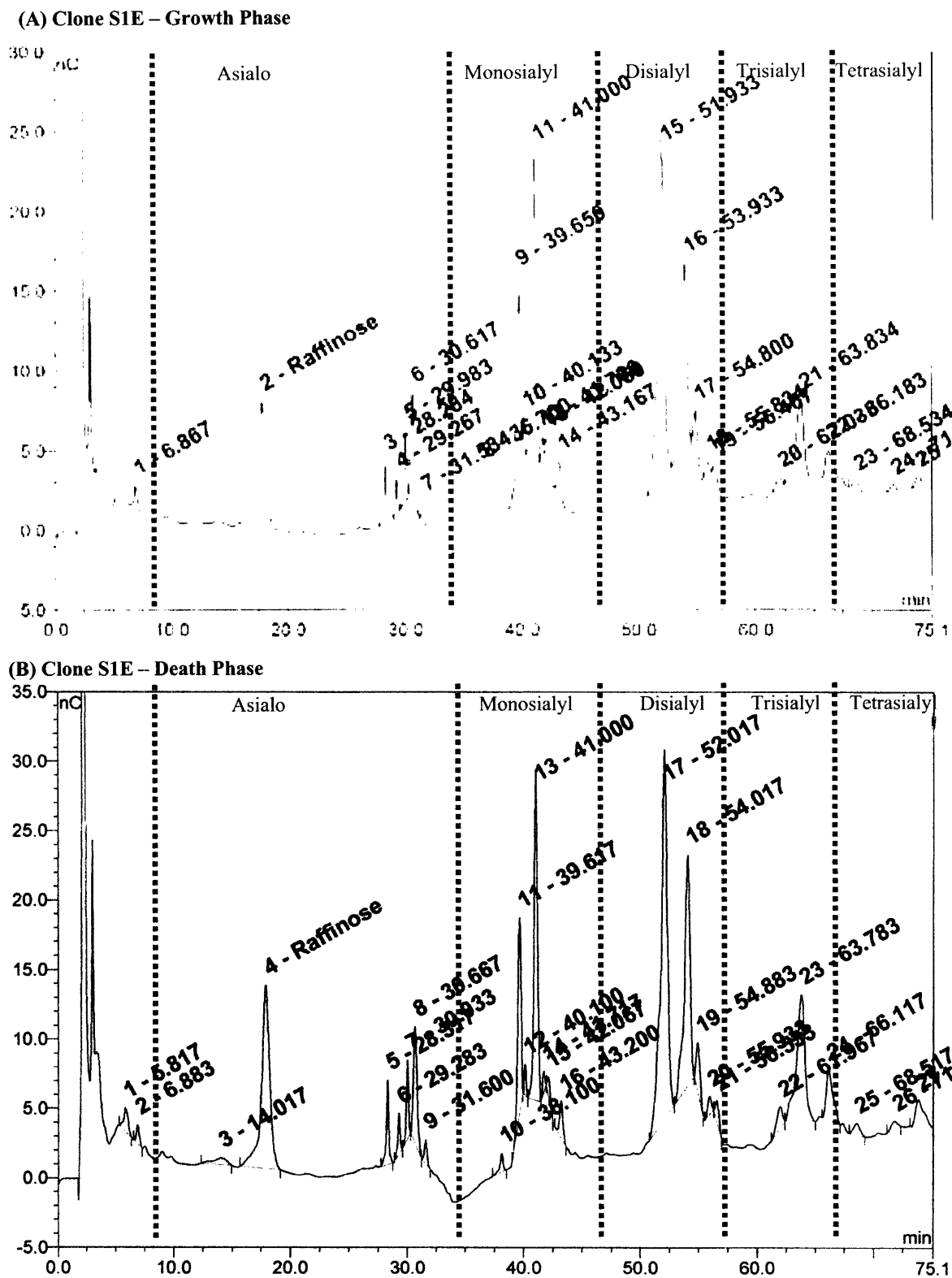


Figure 5.14 High performance anion exchange chromatography spectra of interferon-gamma glycans obtained from clone S1E cell lines during growth phase and death phase.

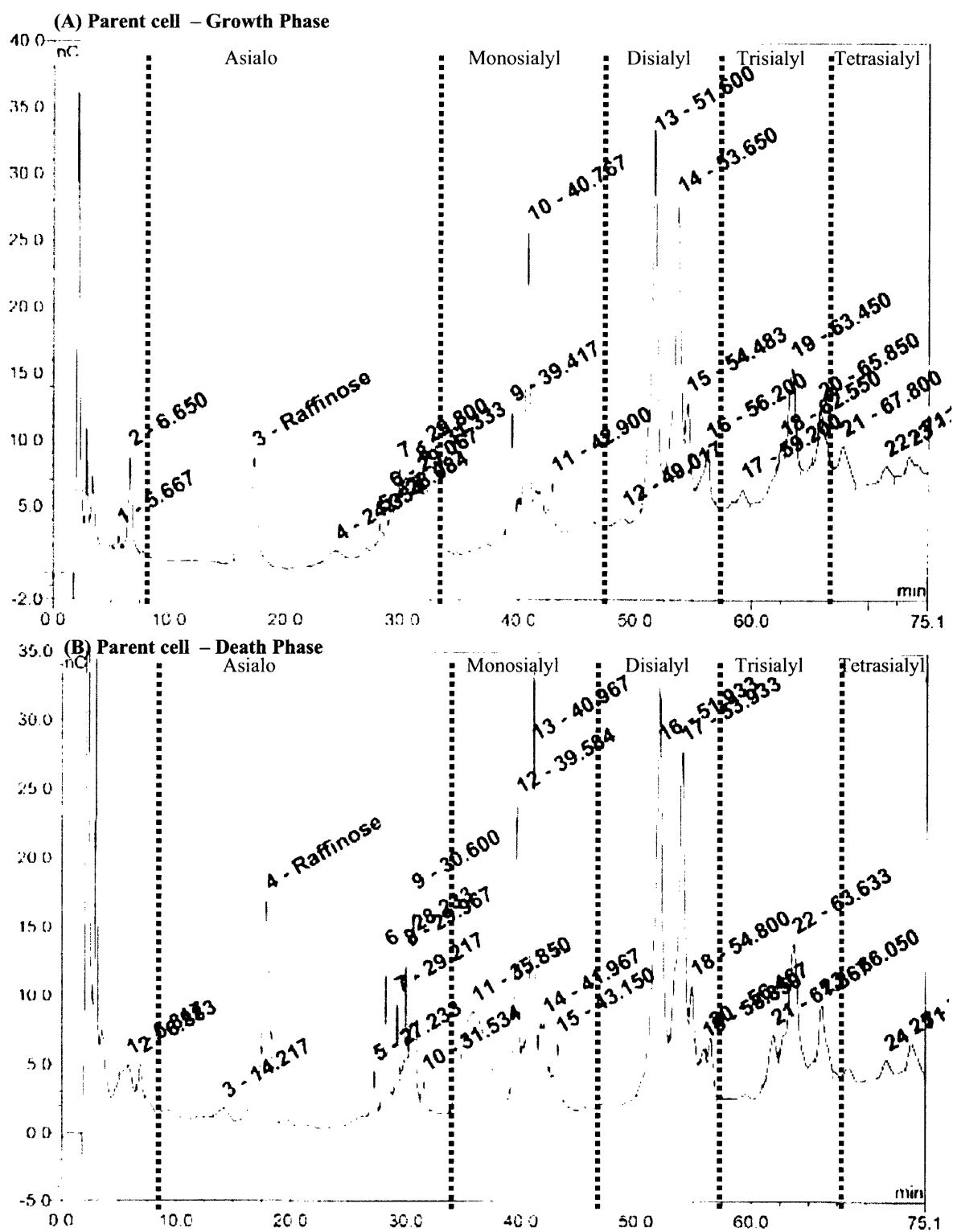


Figure 5.15 High performance anion exchange chromatography spectra of interferon-gamma glycans obtained from parent cell lines during growth phase and death phase.

is impossible without knowing sialylated structures of glycoprotein. Therefore, to estimate percentage of sialylation, integration of HPAEC-PAD resolved peak area was performed.

In our study, we found that biantennary structure represents the most dominant IFN γ glycoform. Thus, sialylation percentage can be readily calculated based on occupancy of the available sialylation sites of biantennary IFN γ glycoform:

$$\text{Sialylation percentage} = \frac{2 \times \text{[Sialylated biantennary structure]} + 1 \times \text{[Fucosylated biantennary structure]}}{2 \times \text{[Sialylated biantennary structure]} + 2 \times \text{[Fucosylated biantennary structure]} + 2 \times \text{[Monosaccharide structure]}} \times 100\%$$

where each figure represented the integrated peak areas of fucosylated and non-fucosylated form obtained from HPAEC-PAD chromatograms. Using this definition, sialylation percentage of IFN γ produced by parent cells and clone S1E could be compared more quantitatively. As shown in Figure 5.16, sialylation percentage of IFN γ produced by clone S1E was relatively constant at 81-83% throughout various phase of cell culture (i.e. growth phase and death phase). This demonstrated the efficacy of sialidase RNAi technique in silencing degradative action of sialidase and maintaining the consistency of high sialic acid content during cell culture. On the other hand, sialylation percentage of IFN γ produced by parent cells decreased from 88% (during growth phase) to 77% (during dead phase) within 240 hours. This corresponded to a decline of sialic acid at the rate of 1%/day due to the degradative action of sialidase released by lysed cells. The sialylation percentage estimated from HPAEC-PAD analysis demonstrated similar trend as the estimate from thiobarbituric acid assay method of quantifying sialic acid content (Figure 5.11).

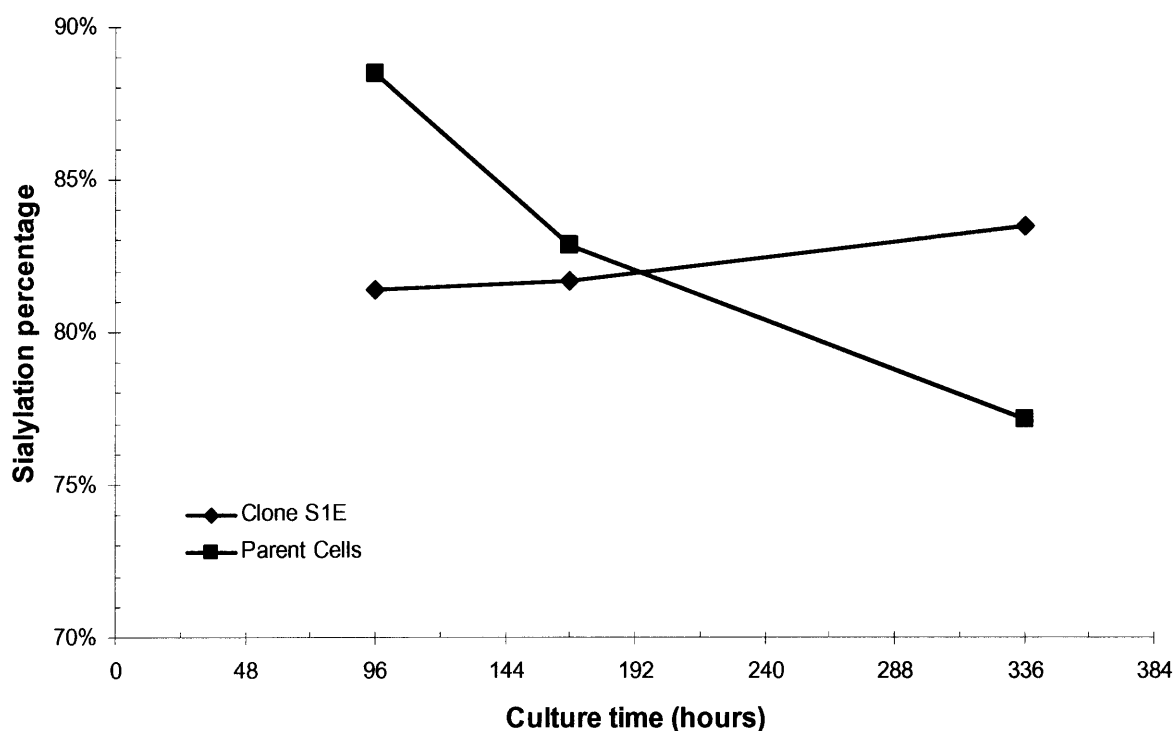


Figure 5.16 Microheterogeneity analysis: Effect of inhibiting sialidase via RNA interference technique on sialylation of complex biantennary interferon gamma glycans in batch CHO cell culture.

Interferon-gamma glycan structures were resolved utilizing high-performance anion exchange chromatography (Figure 5.14 and Figure 5.15). Peaks corresponding to numerous forms of biantennary complex were integrated to obtain a more accurate estimate of sialylation percentage (Gu, 1997).

It was worth noting that the decline in sialylation of parent cells was not as severe as those observed by previous researcher working on the same parent cell line (Gu, 1997). Gu observed a decline of sialic acid at the rate of 4%/day, which was four-times worse than our observed result. We postulated the difference in sialic acid degradation rate was due to the difference in medium composition utilized for the study. Gu utilized serum free media while at this stage of the study, we still supplemented our cell culture with 10% heat-inactivated fetal serum (IFS), which contained many glycoproteins such as fetuin, albumin, and transferrin (Gauthier et al., 1995). It was well known that in addition to *de novo* biosynthesis of sialic acid, salvage pathway to replenish the sialic acid pool existed in mammalian cells (Ferwerda et al., 1981; Mendla et al., 1988; Chigorno, et al., 1996; Santell et al., 1999). Briefly, glycoproteins from serum in supernatant were taken up by cells by endocytosis, degraded, and the sialic acid was recycled for biosynthetic purposes. In the serum-supplemented culture that we performed, fetuin, albumin, and transferrin existed as the source for sialic acid pool replenishment via salvage pathway. Nevertheless, the limited amount of fetuin, albumin, and transferrin in serum was insufficient to contribute consistent highly sialylated IFN γ to balance sialidase degradative action.

A closer look on the distribution of sialylated and asialylated glycoforms reconfirmed the benefit of silencing sialidase degradative action via RNAi (Table 5.5). Clone S1E, which has reduced sialidase activity, consistently maintained the fraction of asialo (17-19%), monosialylated (9-10%), and disialylated (71-74%) biantennary glycans during growth and death phase of cell culture. On the other hand, as the culture phase shifted from growth to death phase, parent cells have lower fraction of disialylated biantennary glycans (from 88% to 65%) accompanied by an increase in monosialylated glycans from 8% to 12% and an increase of asialo glycans by two-

Table 5.5 Biantennary glycan structures of interferon-gamma produced by clone S1E and parent CHO-IFN γ cell lines.

The percentage of each biantennary glycoform was estimated by integrating peak areas of HPAEC-PAD chromatograms. Only biantennary glycoform was considered since the composition of interferon gamma is predominantly biantennary.

Cell line	Culture phase	Fraction of asialo biantennary glycans	Fraction of monosialylated biantennary glycans	Fraction of disialylated biantennary glycans
Clone S1E	Growth	19%	10%	71%
Clone S1E	Death	17%	9%	74%
Parent CHO-IFN γ	Growth	12%	8%	88%
Parent CHO-IFN γ	Death	23%	12%	65%

fold (from 12% to 23%). The increasing amount of low sialic acid-containing glycans in parent cell lines reflected the degradative action of sialidase in cleaving sialic acid off of glycoproteins as soluble sialidase was released by lysed cells. The results from Figure 5.16 and Table 5.5 strongly suggest that the knock down of the sialidase contributes to the decrease in desialylation especially in the death phase where such detrimental event is expressed.

Careful observation on sialic acid content (or sialylation percentage) analysis shown in Figure 5.11 and Figure 5.16 revealed that clone S1E has less maximal sialic acid content during growth phase as compared to parent cell lines. Although IFN γ produced by clone S1E has consistent sialic acid content of 3.1 mol sialic acid/mole IFN γ , this amount was 6% lower than maximal sialic acid content of IFN γ produced by parent cell lines (Figure 5.11). Analysis by integrating peak areas of HPAEC-PAD chromatogram reconfirmed this phenomenon with difference in maximum sialylation percentage by 7% (Figure 5.16). There were two postulates in attempt to explain this phenomenon. First, parent cell lines are population of interferon-gamma producing Chinese hamster ovary cells. Clonal variation exists from generation to generation of parent cells. This reflects on the different level of endogenous sialylation machinery and results in different maximal sialic acid content of IFN γ . Similar cell line used in this study was studied by previous researchers with sialic acid content ranging from 2.3 – 3.1 mole of sialic acid/mole of IFN γ (Gu, 1997).

The second postulate was related to the RNA interference of sialidase degradative action. We proposed that silencing sialidase could lead to less contribution of sialic acid via salvage/recycle pathway. There are two ways of generating sialic acid needed to sialylate glycoprotein (Figure

5.17). The first pathway is the *de novo* biosynthesis pathway (Schauer and Corfield, 1982). Briefly, sialic acid is synthesized in cytoplasm from N-acetylglucosamine by a number of enzymatic reactions before sialic acid is converted to CMP-activated sialic acid (Schauer, 1982). This activated form of sialic acid is then transferred onto the glycans of glycoproteins by the action of sialyltransferase in the Golgi apparatus (Chen et al., 2003). In this pathway, the knock down of sialidase should not alter the amount of sialic acid because sialidase is not involved in *de novo* biosynthesis pathway at any known steps.

The second pathway on the accumulation of cellular sialic acid pool is the salvage/recycle pathway (Figure 5.17). Santell and co-workers (1999) supplemented CHO culture with radioactively-labeled N-acetylmannosamine and observed that when sialic acid contribution from N-acetylmannosamine was reduced to nothing, glycoprotein still contained high amount of sialic acid/mole of protein. This suggested the reutilization of sialic acid originating from glycoprotein existing in the supernatant. The same phenomenon was also observed by other researchers in fibroblasts (Mendla et al., 1988), male rats (Ferwerda et al., 1981), and many other systems. In this pathway, glycoproteins in supernatant are internalized, transported to the lysosomes, and degraded by sialidase (Hollister et al., 2003). The free sialic acid is then converted to CMP-sialic acid before it is being incorporated as terminal sugar in glycoconjugates. In this pathway, without sialidase, sialic acid cannot be extracted from recycled glycoprotein to increase sialic acid pool. Although our studies attempted to specifically knock down cytosolic sialidase, non-specific silencing of other forms of sialidase was still possible. It was known that in other species (such as human), four different forms of sialidase have amino acid sequences which are 75-90% similar (Monti et al., 2002). Since other forms of sialidase in

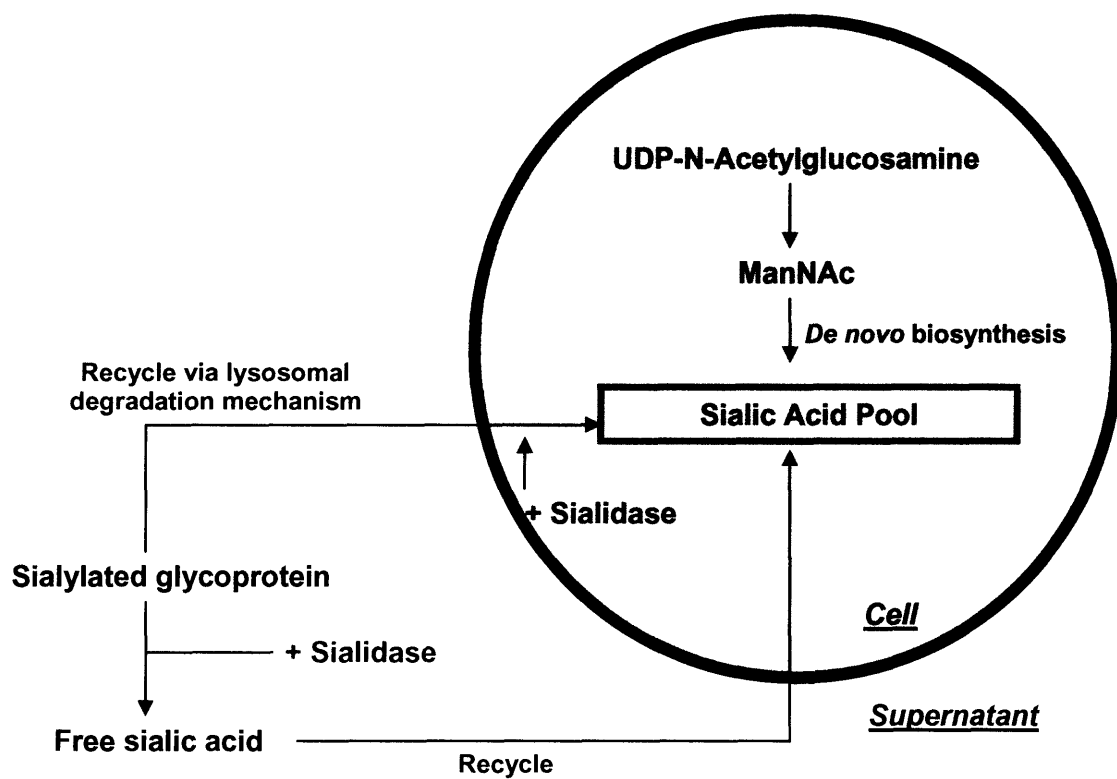


Figure 5.17 Schematic diagram of possible role of sialidase in salvage/recycle pathway of sialic acid.

CHO cells have not been cloned and sequenced, the possibility of knocking down lysosomal sialidase while targeting cytosolic sialidase can't be ruled out. Therefore, if the 21-nt sequence selected to silence cytosolic sialidase overlapped with lysosomal sialidase, contribution of sialic acid from salvage pathway of extracellular glycoprotein would decline and this could lead to reduction in maximal sialic acid content of single cell clones.

In addition to glycoprotein as a source of sialic acid for salvage/recycle pathway, extracellular sialic acid in supernatant could also contribute to sialic acid pool via recycle pathway. Oetke (2001) demonstrated that within a period of 3 days, 2 nmol of free sialic acid per 10^7 cells were incorporated into glycoconjugates of endogenously hyposialylated mammalian cells. Similar results were obtained for NIL, BHK, 3T3 cell lines (Hirschberg et al., 1976; Hirschberg and Yeh, 1977). Free sialic acid in supernatant could be derived from supplementation of sialic acid or from the degradation of glycoprotein in supernatant by soluble cytosolic sialidase (Figure 5.17). If cytosolic sialidase is knocked down, contribution of sialic acid from this recycle pathway will decline, resulting in a reduction in maximal sialic acid content of single cell clones. However, this recycle pathway was not likely to be the dominating factor because cell viability was still very high during the growth phase.

GFP-based Pol III-driven shRNA plasmid: Selection strategy

GFP-based Pol III-driven shRNA plasmid was constructed by site specific recombination of a cloning entry vector and gateway destination vector (Figure 3.8). These vectors were graciously provided by Dr. Iain Fraser (Caltech). Sialidase shRNA sequence was utilized based on the initial design of sialidase siRNA sequence S1 (Table 3.1). CHO cells were transfected with the GFP-based Pol III-driven shRNA plasmid and screened with neomycin. After a few weeks, cells were sorted using FACS and two populations were obtained (Figure 5.18). The high GFP population was defined as those with mean fluorescence intensity higher than 10^2 while the low GFP population (R8 in Figure 6.1) was defined as those with mean fluorescence intensity less than or within the range of $10^0 - 10^1$. We further split the high GFP population into three subpopulations: very high fluorescence subpopulation (R5 in Figure 6.1), high fluorescence subpopulation (R6 in Figure 6.1), and medium fluorescence (R7 in Figure 6.1). R5 has mean fluorescence intensity above 5×10^3 , R6 has mean fluorescence intensity $\sim 2 \times 10^3$, while R7 has mean fluorescence intensity $\sim 4 \times 10^2$. Each subpopulation was expanded in separate T-flasks.

Fluorescence profiles of subpopulations with GFP-based Pol III-driven shRNA plasmid

A hundred thousand cells from each subpopulation were inoculated in six-well plates to investigate the GFP profile of each subpopulation. The same amounts of viable cells were collected every other day to standardize the mean GFP fluorescence per million cells. As shown in Figure 5.19, the mean GFP fluorescence increased as culture time increased. This phenomenon was observed for all subpopulations except for untransfected control cells. The increasing GFP fluorescence for similar amount of cells was contributed due to accumulation of

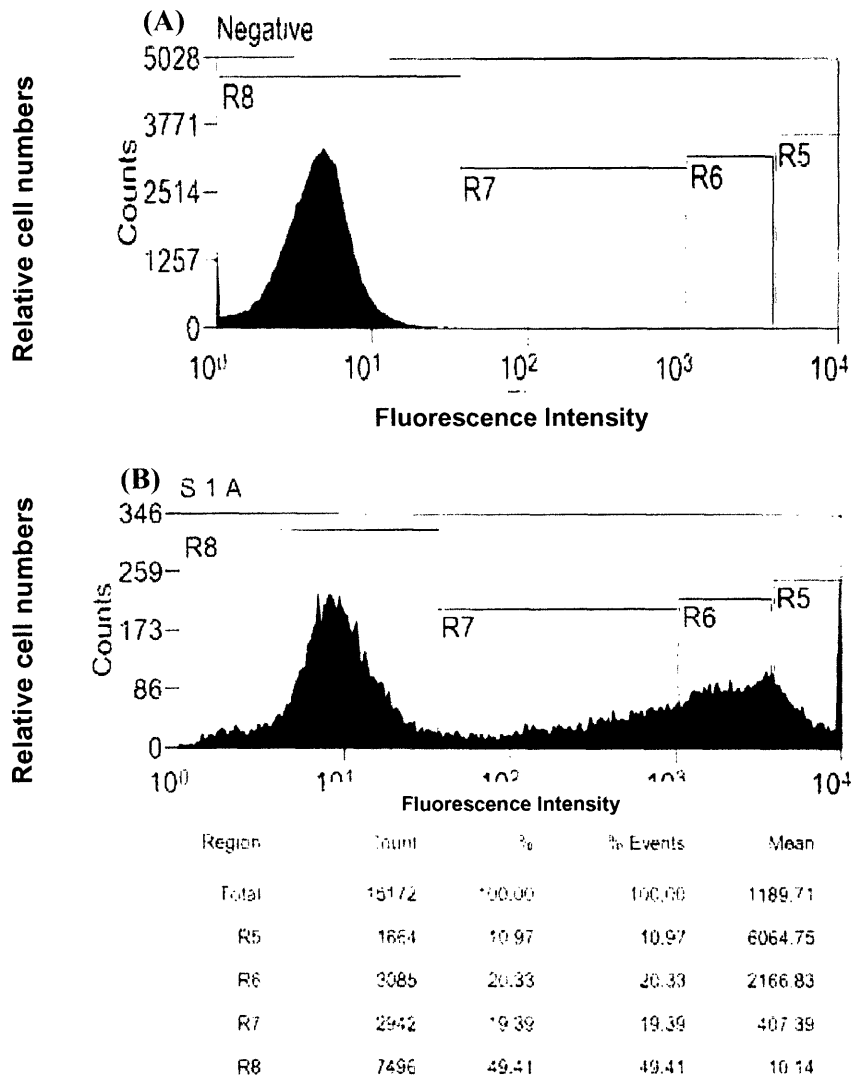


Figure 5.18 FACS analysis of CHO cells transfected with pol III-driven shRNA-GFP plasmid bearing drug resistance marker

CHO cells were transfected with pol III-driven shRNA-GFP plasmid and screened with neomycin. During FACS analysis untransfected CHO-IFN γ cells were used as a negative control (Figure 6.3A). Transfected cells were separated based on the top 10% mean GFP fluorescence (R5 in Figure 6.3B), the next top 20% (R6), the following top 20% (R7), and the bottom 50% (R8).

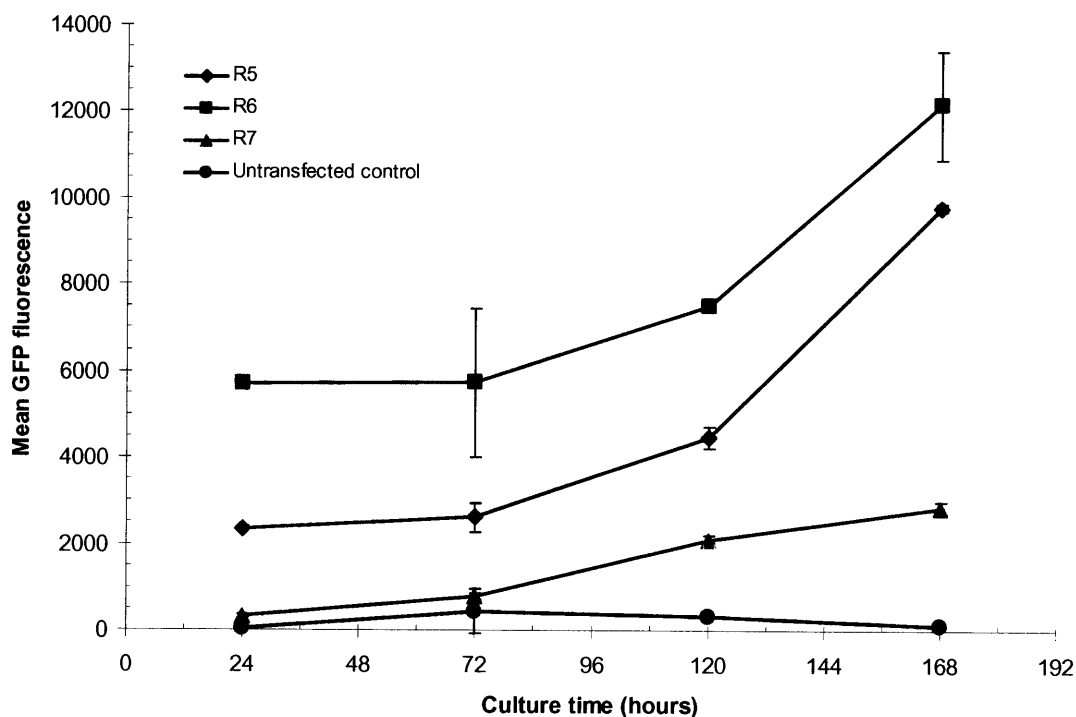


Figure 5.19 Mean GFP fluorescence intensities of selected subpopulations transfected with pol III-driven GFP-based shRNA plasmid and untransfected parent cell lines are compared over the course of cell culture.

Subpopulations obtained from FACS analysis were expanded in T-150 flasks with neomycin selection. 1×10^6 cells were collected every other day and washed a few times with PBS before they were diluted in PBS for mean fluorescence measurements. Error bars represent the differences in fluorescence measurements obtained from triplicate cultures.

GFP protein produced by actively growing cells. This increasing amount of GFP protein should in turn lead to an increasing amount of sialidase hairpin because both the hairpin and GFP construct were located within same plasmid.

Sialidase profiles of subpopulations with GFP-based Pol III-driven shRNA plasmid

Real time RT-PCR and a fluorescence-based sialidase assay were performed to quantitate the level of sialidase reduction within each subpopulation transfected with GFP-based pol III-driven shRNA plasmid. Subpopulation R7, which had a mean fluorescence intensity that was one order of magnitude lower than subpopulation R6, had sialidase mRNA similar to the level of the parent cells (Figure 5.20). This demonstrated no significant knock down in sialidase mRNA of cells with mediocre fluorescence intensity. The lack of sialidase mRNA reduction in subpopulation R7 translated to sialidase activity that was relatively high.

On the other hand, subpopulation R6, which possessed increased mean fluorescence intensity, had sialidase mRNA level knocked down by 2-3 folds. This phenomenon was observed after 72 hours of cell cultivation. Initially, no reduction in mRNA levels of subpopulation R6 was observed possibly due to insufficient synthesis of sialidase siRNA. The reduction in sialidase mRNA level translated to subpopulations with sialidase activity 27-33% lower than parent cells during cell culture (Figure 5.21). In this study we demonstrated that subpopulations with a high mean fluorescence intensity possessed lower sialidase levels as quantitated in mRNA and protein analysis. Correlation between high GFP and low sialidase expression (due to high shRNA expression) suggests that the GFP-based method is a viable option to rapidly and effectively sort cells with high shRNA expression.

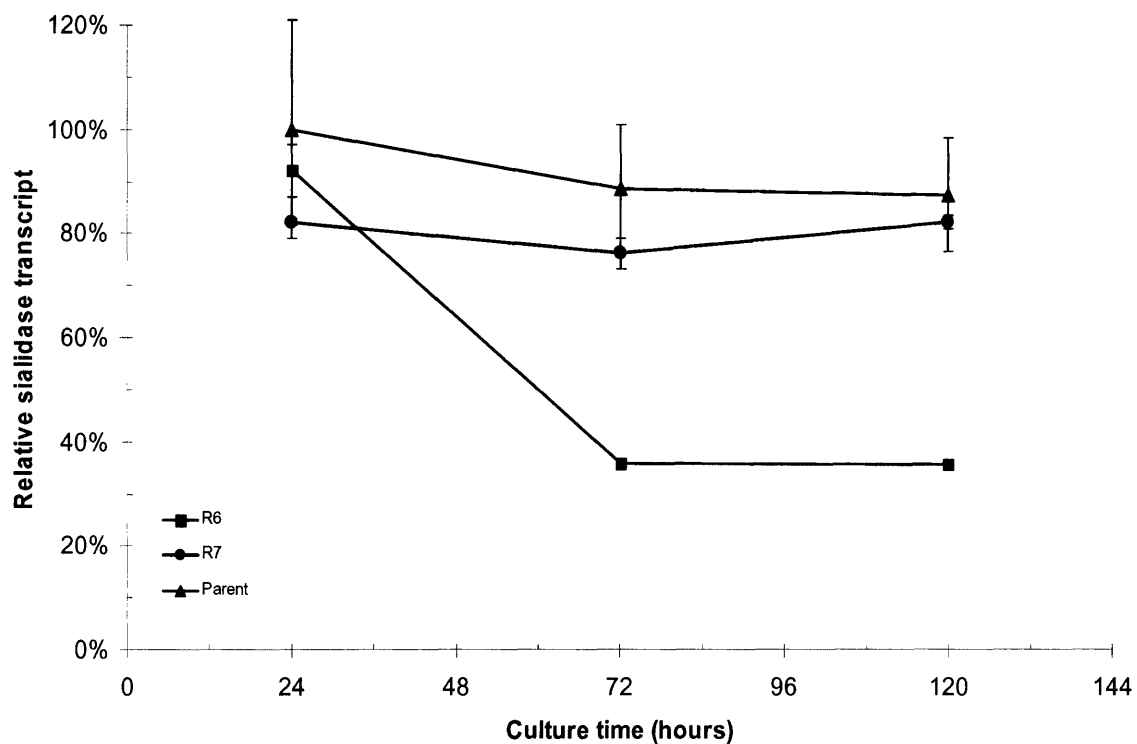


Figure 5.20 Normalized sialidase mRNA level of subpopulations transfected with pol III-driven GFP-based shRNA plasmid obtained from FACS analysis are compared with untransfected parent cell lines.

1×10^6 cells were harvested for RNA isolation. DNase treated RNAs were stored in -80°C before they were used for real time RT-PCR.

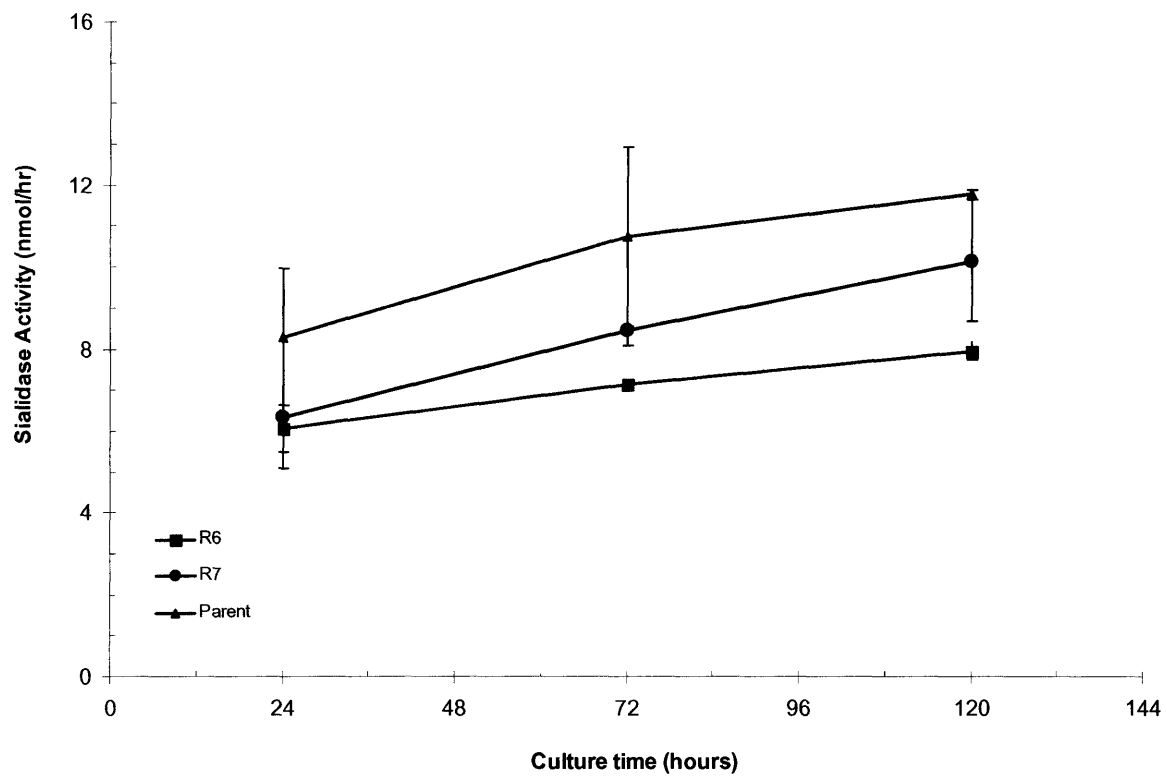


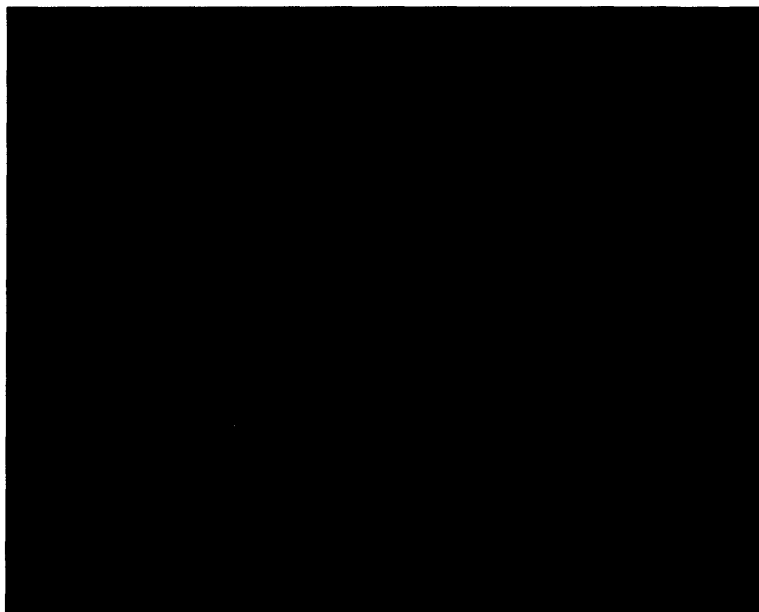
Figure 5.21 Sialidase activity of subpopulations transfected with pol III-driven GFP-based shRNA plasmid obtained from FACS analysis are compared with untransfected parent cell lines

1×10^6 cells were harvested and lysed every other day before they were subjected to a fluorescence-based sialidase activity assay. Enzymatic assay was performed in triplicates.

GFP-based Pol II-driven shRNA plasmid: Design and selection strategy

In the previous chapter, we demonstrated that the Pol II promoter (such as modified CMV promoter) was a much stronger promoter than the Pol III promoter (such as U6 or H1 promoter) in generating shRNA to silence the expression of a gene of interest. Thus the next step of our study was to implement our GFP-based technique to a shRNA plasmid with a stronger promoter such as the modified CMV promoter. We attempted to insert the GFP fragment from pEGFP-C1 (BD Biosciences) plasmid into the pSilencer 4.1-CMV (Ambion) plasmid. The GFP fragment amplified from pEGFP-C1 plasmid included its own promoter because the promoter for shRNA was not the same as that for the heterologous protein marker. Fluorescent microscopy was performed to test the success in integrating the GFP fragment to the shRNA plasmid. Figure 5.22 (A) shows the CHO-IFN- γ transfected with pEGFP-C1 plasmid which served as positive control. Figure 5.22 (B) shows the CHO cells transfected with the engineered plasmid. There were fewer cells with variable levels of GFP intensity as compared to positive control. This was possibly due to the drug screening performed on CHO cells transfected with the engineered plasmid rather than due to the failure in combining both plasmids. DNA sequencing was performed on the engineered plasmid and the result confirmed the existence of GFP fragments within the pSilencer 4.1-CMV plasmid.

(A) CHO-IFN γ cells transfected with pEGFP-C1 plasmid



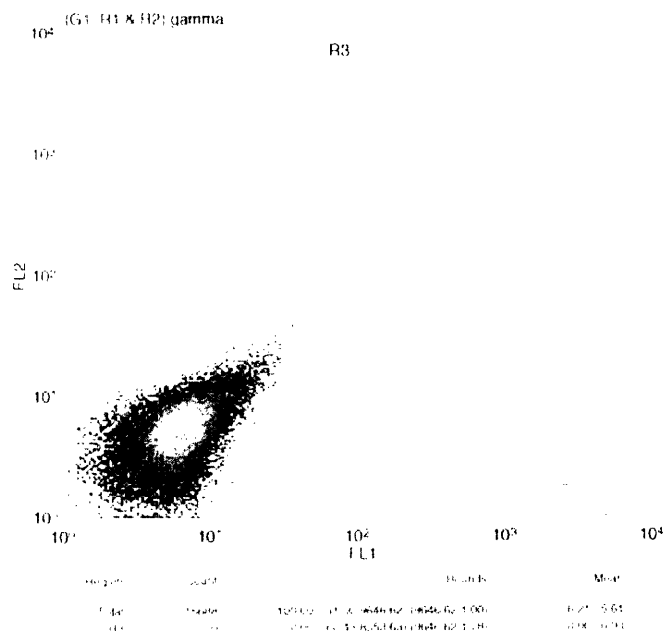
(B) CHO-IFN γ cells transfected with modified pSilencer 4.1-CMV plasmid



Figure 5.22 Fluorescence microscopy images of CHO cells transfected with pEGFP-C1 plasmid (A) and with modified pSilencer 4.1-CMV plasmid (B) are compared.

pSilencer 4.1-CMV plasmid was modified to contain a fragment of GFP sequence from pEGFP-C1 plasmid. The success in plasmid engineering was tested by checking the fluorescence of transfected CHO cells.

(A) Negative control: untransfected CHO-IFN- γ



(B) CHO-IFN- γ transfected with modified pSilencer 4.1-CMV (Figure 6.2)

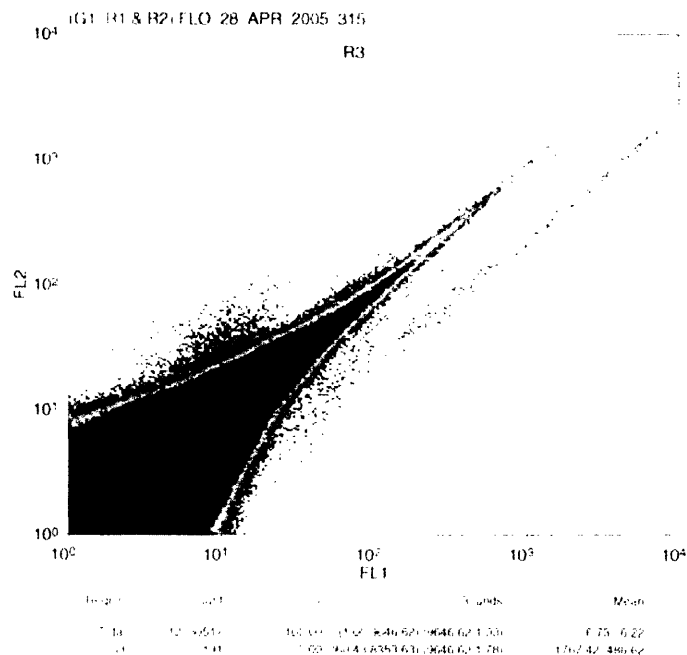


Figure 5.23 FACS analysis of CHO cells transfected with pol II-driven shRNA-GFP plasmid bearing drug resistance marker

Fluorescence and sialidase profiles of subpopulations with GFP-based Pol II-driven shRNA plasmid

FACS analysis was performed on CHO cells transfected with the GFP-based pSilencer 4.1-CMV plasmid (Figure 5.23). Only about 191 cells possessed significant fluorescence intensity out of the 1.2×10^7 cells that were drug resistant. This demonstrated that drug resistant cells that resulted from plasmid transfection did not guarantee the possession of a high amount of plasmid integrated within the genome. FACS analysis can help obtain cells that are drug resistant and that possess a high mean GFP intensity, which should correspond to colonies with high sialidase shRNA expression. The increasing GFP fluorescence as a function of culture time was observed (Figure 5.24). This was in agreement with previous findings with different promoters (Figure 5.19).

Significant sialidase knock down was observed in sorted cells. Sialidase mRNA levels of transfected cells were lower by 3-6 folds than those of parent cells (Figure 5.25). This led to a 62-77% reduction in sialidase activity (Figure 5.26). It is worth noting that mean GFP intensity of Pol II-based colonies was similar to that of Pol III-based subpopulations (Figure 5.19 and Figure 5.24). However, the sialidase mRNA reduction in Pol II-based colonies was stronger by 1-3 folds (Figure 5.20 and Figure 5.25). This supported our hypothesis that the Pol II promoter was a better promoter than the Pol III promoter in knocking down genes pertinent to CHO cell applications.

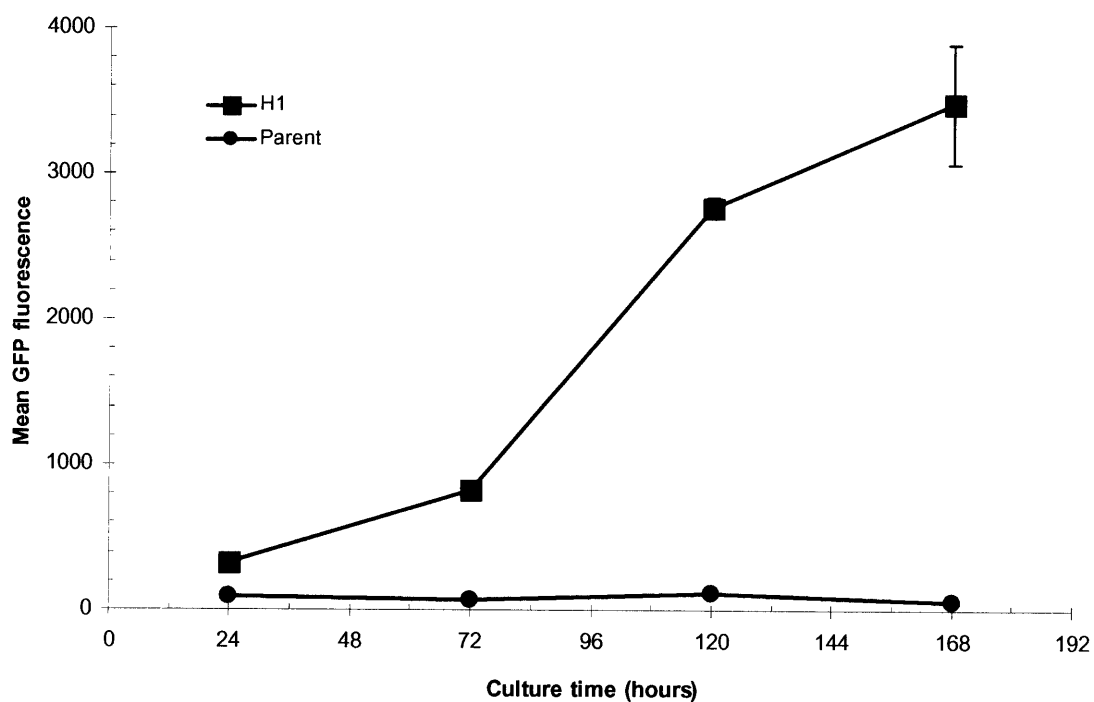


Figure 5.24 Mean GFP fluorescence intensities of selected subpopulations transfected with pol II-driven GFP-based shRNA plasmid and untransfected parent cell lines are compared over the course of cell culture.

Subpopulations obtained from FACS analysis (Figure 5.23) were expanded in 6-well plate, T-75 flasks, and T-150 flasks with hygromycin selection. 1×10^6 cells were collected every other day and washed a few times with PBS before they were diluted in PBS for mean fluorescence measurements. Error bars represent the differences in fluorescence measurements obtained from triplicate cultures.

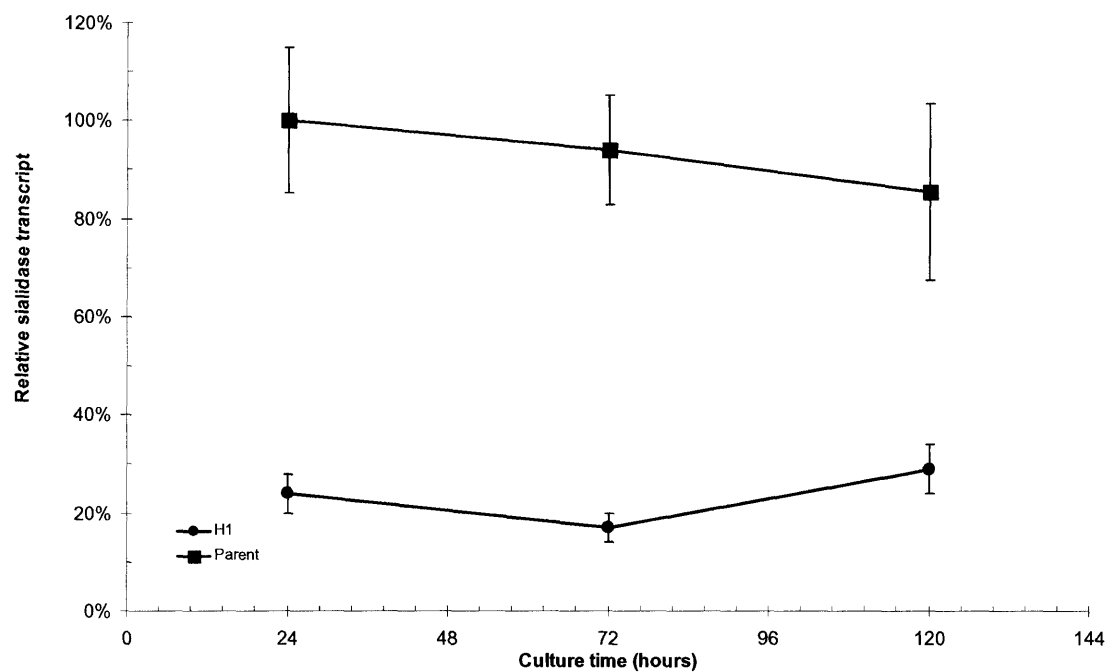


Figure 5.25 Normalized sialidase mRNA level of subpopulations transfected with pol II-driven GFP-based shRNA plasmid obtained from FACS analyses (Figure 5.23) are compared with untransfected parent cell lines.

1×10^6 cells were harvested for RNA isolation. DNase treated RNAs were stored in -80°C before they were used for real time RT-PCR.

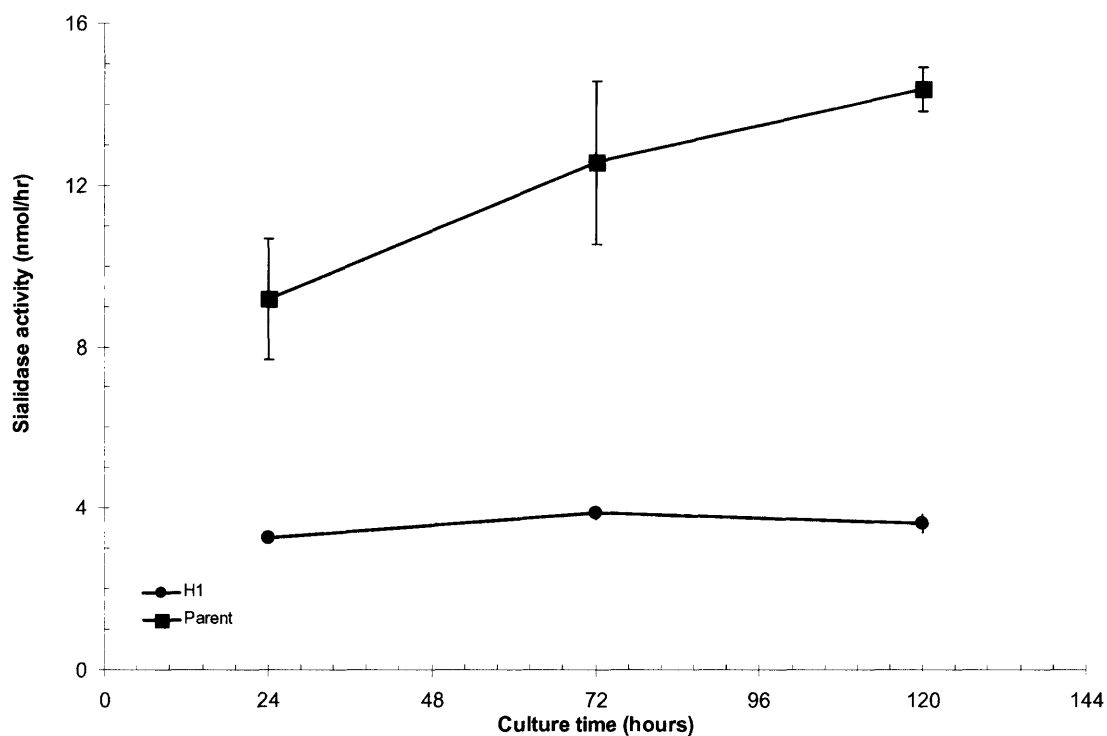


Figure 5.26 Sialidase activity of subpopulations transfected with pol II-driven GFP-based shRNA plasmid obtained from FACS analyses (Figure 5.23) are compared with untransfected parent cell lines.

1×10^6 cells were harvested and lysed every other day before they were subjected to a fluorescence-based sialidase activity assay. Enzymatic assay was performed in triplicates.

5.4 Discussions

Heterogeneity in the glycoforms of proteins is a function of the types of organism used as production host and culture condition. A better understanding of the origins of glycoforms and how to control them are becoming more important because the activity and efficacy of therapeutic recombinant proteins strongly correlates to the structure of the glycoprotein. In addition, from a policy perspective, the Food and Drug Administration (FDA) in the United States and Committee for Proprietary Medical Productions (CPMP) in Europe have demanded consistent carbohydrate structure of therapeutic glycoproteins for human therapy (Liu, 1992).

To control the glycoform, culture optimization and genetic engineering approaches have been attempted. The basis of these works was to either increase intracellular sialylation or to decrease extracellular desialylation by degradative enzymes. In increasing intracellular sialylation, α 2,3-sialyltransferase and/or α 2,6-sialyltransferase have been over expressed in CHO cells (Fukuta et al., 2000; Bragonzi et al., 2001). A slight improvement from 1.12 to 1.16 mole sialic acid/mole IFN γ protein was observed by Bragonzi et al., while improvement of sialylation extent from 61.2% to 79.8% in IFN γ produced by CHO was observed by Fukuta et al. Overexpressing both α 2,3 and α 2,6-sialyltransferase was done by Fukuta et al. (2000) and an improvement of 20-25% in extent of sialylation in IFN γ was obtained. Another way to improve intracellular sialylation was to provide an excess amount of pre-cursor sugars needed in intracellular sialic acid synthesis. Gu and Wang (1998) demonstrated that supplementation of N-acetylmannosamine (ManNAc) up to 20 mM led to 15% improvement in sialic acid content.

Another approach to control glycoform is to decrease extracellular desialylation by sialidase. Addition of sialidase inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (2,3D) was an effective way to maintain sialic acid consistency throughout cell culture. In IFN γ -producing CHO cultures, it was observed that 15% reduction in sialic acid content during death phase could be prevented by supplementing 1 mM 2,3D at the beginning and at 72 hours of cell culture (Gu et al., 1997). Similar results were demonstrated by Gramer et al. (1995) when 1 mM 2,3-D was added to the gp120-producing CHO cell culture. Adoption of this method for an industrial-relevant process was unacceptable due to the high cost incurred for large scale process. With the advent of RNA interference (RNAi) technique, a better way to silence sialidase activity is now possible. The essence of the RNAi technique is to knock down instead of to knock out. Studies on murine B16 melanoma cells demonstrated the relationship between sialidase and pulmonary metastasis (Tokuyama et al., 1997; Kato et al., 2001). Another studies on rat indicated the possible involvement of cytosolic sialidase in the myoblast differentiation (Sato and Miyagi, 1996). These studies strengthened our hypothesis that knocking down sialidase via the RNAi method is a better platform than knocking out sialidase DNA completely.

Utilizing the RNAi technique to knock down sialidase activity, we demonstrated that stable CHO cell clones with reduced sialidase activity can be isolated. Further, the reduction in sialidase activity translated to consistent sialic acid content capping each glycoprotein throughout various cell culture phases. Substantial sialic acid reduction due to sialidase action was not observed during prolonged cell culture. This result is comparable to the sialylated glycoprotein profile when chemically synthesized inhibitor was added extracellularly (Gu et al., 1997). It is worth noting that knocking down sialidase does not improve maximum sialic acid content. Interferon- γ

has maximal theoretical sialic acid content of 4 mole of sialic acid/mole interferon- γ . In our studies, maximal sialic acid during growth phase was about 3-3.3 mole of sialic acid/mole interferon- γ . This range signified incomplete intracellular sialylation rather than sialidase degradative action because viability is >95% during growth phase. Sialic acid synthesis is performed intracellularly by the concerted action of various transferases and sugar nucleotides while sialic acid degradation occurs extracellularly by sialidase activity. Due to these two distinct unrelated pathways, the best approach to have cell lines that produce consistently high sialic acid content glycoprotein to combine genetic engineering/cell culture modification approach to increase intracellular sialylation with RNA interference approach to reduce extracellular desialylation.

This chapter also presents a successful GFP-based strategy to isolate cells that express a high amount of sialidase shRNA plasmid without the needs of testing thousands of clones. The GFP-based shRNA plasmid was constructed either by site specific recombination of two plasmids or by inserting and ligating GFP fragments to a plasmid. Subpopulations of CHO cells with high level of mean fluorescence intensity were shown to possess lower sialidase mRNA level and activity. This implied a positive correlation between GFP fluorescence intensities and siRNA generated to silence sialidase. Further, we also demonstrated that for similar fluorescent intensity, cells transfected with Pol II-based plasmid exhibited much stronger sialidase knock down as compared to cells transfected with Pol III-based plasmid. We believe the same strategy can be applied to knock down other genes, such as those related to apoptotic pathways, and to rapidly isolate cells that express high amounts of gene-specific siRNAs.

5.5 Conclusions

We have established a novel siRNA-mediated sialidase-silencing method for maintaining sialic acid content of IFN γ glycans throughout cell culture. Normally following cell lysis, sialidase is released into the supernatant which results in the cleavage of sialic acids from the glycans on the glycoproteins. By using an RNAi method to inhibit sialidase expression, external sialidase inhibitor was not required nor was a purification method required to remove inhibitor from the cell culture. This results in a more economically efficient process wherein sialic acid content is retained via genetic engineering of the glycoprotein-producing mammalian cells. Using the RNAi method, sialidase activity was reduced significantly (maximal reduction over 60% in growth phase of clone S1E and S5F) and sialic acid content was sustained at a relatively high level (3.1 mole sialic acid/mole IFN γ for clone S1E and 3.3 mole sialic acid/mole IFN γ for clone S5F) throughout the death phase without altering cell metabolism in a deleterious manner. It is our belief that this method for improving glycoprotein quality as measured by high sialic acid content is applicable to other glycoproteins.

5.6 Acknowledgments

I would like to thank Niki SC Wong (Singapore-MIT Alliance) for her kind assistance with the TAA assay and macroheterogeneity analysis. I sincerely appreciate the very invaluable IFN γ microheterogeneity analysis performed by Dr. Goh Lin-Tang, Dr. Lee May May and research scientists at Bioprocessing Technology Institute, Singapore.

6. Conclusions and Recommendations

6.1 Thesis Conclusions

Sialic acids at the termini of the oligosaccharide chains in glycoproteins have been shown to dramatically affect circulatory half life of therapeutic proteins. Unfortunately, during prolonged cell cultivation, lysed cells release sialidases that can cleave sialic acid off from the glycans on the glycoproteins. As a result, glycoproteins with variable terminal sialic acid content are produced. In the past, chemically synthesized inhibitors were added to supernatant to avoid desialylation problems. Unfortunately, this method is not that desirable for industrial processes due to the FDA requirements to ensure complete removal of additives.

In this thesis, we have reconfirmed the existence of sialidase in IFN γ producing CHO cells culture. This was followed by the successful identification of the sialidase siRNA sequences that could transiently knock down sialidase mRNA by 9 folds and accompanied by the reduction of sialidase activity by 4 folds. The most potent sialidase siRNA was found through trial and error and did not follow the widely-used Tuschl's rule. In addition, we have found that the efficacy of numerous sialidase siRNAs utilized in our study did not exactly follow the thermodynamic rules proposed by many researchers.

Pol III promoters such as U6 and H1 are popular promoters for generating siRNA permanently in various cell lines. These promoters were not found to be strong enough to generate sialidase siRNAs in CHO cultures. Modified CMV promoters were more appropriate for knocking down sialidase activity as clones with over 50% sialidase activity reduction could be isolated.

Knocking down sialidase activity did not affect cell viability and heterologous glycoprotein titers. Sialic acid content was relatively constant throughout various phases of prolonged cell cultures. Microheterogeneity analysis reconfirmed the consistent fraction of asialo, monosialyl, and bisialyl form of IFN γ for cell lines with reduced sialidase level. On the other hand, parent cells were found to have more asialo and monosialyl form of IFN γ as the cells died demonstrating the effect of sialidase release on glycoproteins during prolonged cell culture. Maximal sialic acid content during growth phase was found to be affected by sialidase knock down. This could be due to clonal variation of parent cells or due to sialic acid salvage pathway disruption by reduced sialidase. Our results further showed that during prolonged cell cultivation accompanied by cell death, the knock down sialidase cells maintained constant sialic acid contents while the parent cell line indicated a continual decrease in the total sialic content.

Selection of single cell clones expressing high level of sialidase siRNA was the rate-limiting step in creating cells with reduced sialidase level because drug resistance does not guarantee high degree of plasmid integration. Thus, we developed a GFP-based method to reduce the amount of time needed to locate cells which express high amounts of sialidase siRNAs. GFP-based shRNA plasmid was constructed either by site specific recombination of two plasmids or by inserting and ligating GFP fragments into a plasmid. Subpopulations of CHO cells with a high level of mean fluorescence intensity were shown to possess lower sialidase mRNA level and activity. This implied a positive correlation between GFP fluorescence intensity and siRNA generated to silence sialidase. Further, we also demonstrated that for similar fluorescent intensity, cells transfected with Pol II-based plasmid exhibited much stronger sialidase knock down as compared to cells transfected with Pol III-based plasmid.

6.2 Recommendations for Future Work

Fed-batch culture of clones with reduced sialidase level

Batch and fed-batch cultures are two of the most commonly used methods to grow mammalian cells and to harvest biopharmaceuticals. In this thesis, batch culture of CHO-IFN- γ was used as the model system and characterizations of cell lines and glycoproteins were performed extensively. Fed-batch systems yield significant amount of glycoproteins due to prolonged viability (Xie et al., 1997). A detailed study using cell lines that have been transfected with sialidase siRNA in fed-batch system would be the logical continuation of this project because high quality protein produced in high quantity is the ideal platform that could be translated to industrial applications.

In mammalian cell culture, one fed-batch strategy is to feed the amount of the required glucose and glutamine without over feeding these nutrients. In particular, low level of glucose and glutamine are desired because excessive glucose and glutamine would be converted to undesired metabolites such as lactate and ammonia (Wong et al., 2005). With reduced amount of toxic metabolites, cell culture time can be extended. At prolonged culture time, cells transfected with sialidase siRNA may no longer have major desialylation problems due to extracellular degradation. If incomplete sialylation is observed, it is very likely that incomplete intracellular sialylation is the major contributor of low sialic acid containing glycoproteins. Microarray studies comparing transcript profiles of parent cells and CHO cells transfected with sialidase siRNA could reveal what genes are being up regulated or down regulated during fed batch culture.

For clones with reduced sialidase level, another potential supplement during the fed batch strategy was the sialic acid and/or N-acetylmannosamine. Mammalian cells are capable of taking up and incorporating sialic acid onto the glycoproteins (Hirschberg et al., 1976; Hirschberg and Yeh, 1977; Ferwerda et al., 1981; Mendla et al., 1988; Oetke et al., 2001). The supplementation of N-acetylmannosamine was demonstrated to increase sialylation percentage by about 15% (Gu and Wang, 1998). By feeding sugar and sugar precursors needed for sialylation on the cells with reduced sialidase level, it is expected that glycoprotein with a consistent and very high amount of sialic acid could be achieved.

Silencing sialidase mRNA on mammalian cells that produce heavily glycosylated proteins

In this study, IFN γ produced by CHO cells was used as a model because characterization techniques on this glycoprotein have been established by many researchers in our laboratory (Gu, 1998). To substantiate the generality of our sialidase siRNA technique to improve sialic acid consistency, it is imperative to attempt to silence sialidase mRNA on mammalian cells that produce heavily glycosylated/sialylated proteins. IFN γ only has 4 maximal terminal sialic acids. As a result, sialylation improvement during prolonged cell culture might not be as dramatic as knocking down sialidase on cells that produce darbepoetin alfa (which has maximal sialic acid of 22 mol/mol proteins) or Factor VIII proteins (which has typical sialic acid content of 30 mol/mol protein) (Sodetz et al., 1977; Catlin et al., 2002).

Creation of universal cell line CHO-DG44 with reduced sialidase expression

In our study, we have constructed IFN γ -producing CHO cells that have reduced sialidase activity level. Unfortunately, the cell lines produced by our lab could not be used to produce other

heterologous glycoproteins via methotrexate amplification because IFN γ was co-amplified with DHFR gene. A useful cell line to be used as a platform for other industrial-relevant glycoproteins is CHO-DG44 that is transfected with sialidase siRNA plasmid. CHO-DG44 is the universal cell line that is typically used as a starting point to construct heterologous protein producing cell lines (Urlaub et al., 1986). By having a CHO-DG44 that has reduced sialidase level via RNAi method any glycoprotein, in theory, could be produced without the threat of extracellular desialylation. The GFP-based method that was developed in this thesis could be used to rapidly produce CHO-DG44 with reduced sialidase expression.

Examining hybridoma cell lines with reduced glycosidase expression

The use of hybridoma cells is also practiced in industrial processes for monoclonal antibody production (Chu and Robinson, 2001; Ibarra et al. 2003). In particular, when large amount of antibodies is required, prolonged fed-batch cultivation will be employed. Normally following cell lysis, glycosidase enzymes are released into the supernatant which results in the cleavage of functional sugar from the glycans on the glycoproteins. It is recommended that the principles and techniques developed from this thesis be tested in hybridoma cells to assess the generality of siRNA as a mean to reduce glycosidase expression.

Other applications using siRNA knock down in mammalian cell cultures

There are various deleterious enzymatic reactions in the metabolic pathways of mammalian cells that would be desirable for elimination. For example, the production of lactic acid due to the lactic acid dehydrogenase is a common undesirable feature in mammalian cell metabolism (Glacken et al., 1986; Kromenaker and Srienc, 1994). The formation of ammonia due to

glutamine deaminases is another example where the end product (ammonia) can affect the growth, product formation, and product quality in mammalian cells (Hansen and Emborg, 1994; Andersen and Goochee, 1995; Capiumont et al., 1995). It is our belief that the methods developed using siRNA knock down are ideally suited to reduce these deleterious enzymatic reactions and is therefore recommended for future study.

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